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TRYPARSAMIDE IN SLEEPING SICKNESS.*

A STUDY OF FORTY CASES, WITH SPECIAL REFERENCE TO THE CEREBROSPINAL FLUID.

BY CLEMENT C. CHESTERMAN, O.B.E., M.D. (Lond.), D.T.M. & H. (Cantab.)

The work summarised in this paper was carried out between the months of August, 1921, and September, 1922, by the author, at the *Stapleton Memorial Hospital* of the Baptist Missionary Society at Yakusu, near Stanleyville, Belgian Congo.

Had it not been that one was single-handed, and fully occupied with many duties largely unconnected with the surgical and medical practice of a small though busy hospital, these investigations could have been carried out in a much more efficient way, and would have furnished reliable data for many more conclusions than can be deduced from the work as it stands. It is satisfactory to know, however, that the cases reported are being watched by my colleague, Dr. F. Gordon Spear, and that either before or after my return to Congo in the autumn, it will be possible to publish a second report on the subsequent progress, the longest record of which, up to date, is but eleven months.

I wish, at the outset, to acknowledge with cordial thanks the goodwill and practical help of the Belgian State officials, and the officers of the Colonial Medical Service, to which I had the honour of being attached in the capacity of *médecin agréé*, a position which, while allowing one the utmost freedom, at the same time secured a very practical co-operation and the note of authority, frequently indispensable, in spite of the utmost confidence of the natives living in the territory in the vicinity of the Mission under my supervision. I would also thank my non medical colleagues for their forbearance in allowing the practically constant presence of advanced cases of sleeping sickness within the station precincts, a fact that accounted for not a few amusing (in retrospect) incidents.

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* The work reported in this paper represents an extension of the chemotherapeutic investigations carried out in The Rockefeller Institute for Medical Research. The drug used for the work was supplied by the Institute.

Tryparsamide.—Sodium salt of N.-Phenylglycineamide-*p*-Arsonic Acid. This drug was first prepared by Jacobs and Heidelberger, of The Rockefeller Institute for Medical Research, and an account of it and related compounds, all synthesized from the German product, phenylglycine-*p*-arsonic acid, was published in 1919,[1] although originally made and studied in 1915.

The action of this drug on trypanosome and spirochæte infections of the smaller laboratory animals was studied by Drs. Wade H. Brown and Louise Pearce, and the results published in 1919.[2]

The encouraging results obtained decided The Rockefeller Institute to send a mission to the Belgian Congo, and Dr. Louise Pearce has embodied the results of her work there in the latter half of 1920 in a masterly treatise.[3] It was there, at the laboratory of Léopoldville, that the author had the privilege of becoming acquainted with this extremely capable worker, and of seeing some of the results of the treatment of human trypanosomiasis with tryparsamide. Before publication of her results, in December, 1921, Dr. Pearce sent me a supply of the drug, as did Dr. Van den Branden, Director of the Léopoldville laboratory, with the request that I should undertake further research on the result of repeated administration in the endeavour to render normal the cerebrospinal fluid of advanced cases. It is, then, with very great pleasure that I acknowledge my indebtedness to Dr. Louise Pearce for the valuable information and suggestions which she has given me, and to The Rockefeller Institute, and Dr. Flexner in particular, for the continued supply of tryparsamide.

Object. The superiority of tryparsamide over atoxyl and antimony compounds, both in the speed with which peripheral sterilisation was produced, and the duration thereof, was demonstrated by Dr. Pearce's work, and its effect in diminishing rapidly the cell content of the cerebrospinal fluid *pari passu*, with marked clinical improvement in advanced cases, was also brought out. But, for the most part, short courses were given, and the total administered did not exceed an average of 12 grammes.

This work was undertaken, therefore, to test the results of repeated administration of large doses at weekly intervals by the intravenous route, with the object of rendering normal the cell content of the cerebrospinal fluid of the most advanced cases.

Material.—The forty cases reported were chosen from among the 300 odd cases diagnosed during the period, for the following reasons:—

(*a*) The source of their infection was known.

(*b*) They showed definite symptoms of nervous involvement and altered cerebrospinal fluid.

(*c*) They would consent to remain in the lazaret, *i.e.* in an area where there was no possibility for re-infection by tsetse flies during treatment.

They comprise men, women and children, all natives; many had to be carried to the hospital in the first instance, others had for some time given up their work on account of the disease.

I have done no inoculation experiments into animals in order to determine the species of trypanosome with which we have been dealing, but I have had no cause to suspect that it is other than *Trypanosoma gambiense*. In view of a case having been recently reported[4] from the Sudan of human infection with *T. rhodesiense*, however, the question is open to dispute.

In thirty-one out of the forty cases an obvious clinical diagnosis was confirmed by the finding of trypanosomes in the gland juice, blood (after centrifugalisation), or cerebrospinal fluid, or in combinations of these. In the remaining nine, the diagnosis was confirmed by a pathological increase in the cell content of the cerebrospinal fluid, no time being wasted in lengthy blood examinations, if this last criterion was forthcoming.

Routine Procedure. Investigation, after gland puncture, commenced with lumbar puncture. The patient was seated on a low stool with head bowed on the arms resting on a low table. In the first cases, when it was definitely desired to establish the presence of trypanosomes in the fluid, 10 c c. was withdrawn, but in later investigations, a few drops sufficed. The patient was then placed in bed, and after periods ranging from one to twenty-four hours, the first intravenous injection of tryparsamide was given. This was given in a solution made up with boiled rain water, of a concentration equal to 3 gm. in 10 c.c.

A course of eight weekly injections was regarded as the normal, but in some cases this had to be varied on account of the development of visual disturbance, when both the dose and the frequency, or one of the two, was altered, according to the changes in visual acuity.

At intervals varying from one week to seventeen weeks after the end of the course the second puncture was performed. Subsequent examinations will, it is hoped, form the subject of a further report.

Examination of the Cerebrospinal Fluid.—Examination, except in those cases in which 10 c.c. was withdrawn and trypanosomes looked for after centrifugalisation, was confined to a count of the cells present, using a Thoma-Zeiss ruled slide fitted with a coverslip so as to produce the ordinary layer of liquid of a depth of $\frac{1}{10}$ mm. As the fluid was examined undiluted, and the count made by the ⅔ in. objective, after careful examination by the ⅙ in. objective so as to exclude the red blood corpuscles, the whole ruled area was visible in the field, and multiplication of the number of cells counted in the ruled area by 10 gave the number per cubic millimetre. Two counts only were made, as a rule, in specimens showing a marked increase, but in specimens where cells were scanty, careful comparison of the density of cells in neighbouring fields was made in addition so as to ensure greater accuracy.

I fully agree with the value of the cell count as a safe guide to the degree of nervous involvement of the patient, as has been universally accepted after the researches of Broden and Rodhain.[5]

The presence of large mononuclears and the degenerate so-called "mulberry" cells, was noted in practically all cases, but no special record of them was made. The presence also of trypanosomes in the fluid is of little consequence, and probably as variable as it is in the blood.

An average of not more than one cell in two fields (ruled areas) was considered normal, *i.e.* 5 per c.mm.

Results.—These are given in the Tables I., II., and III., with brief clinical notes on each case following. Table I. contains the record of twenty-four cases who had received no previous treatment with any drug. Table II. shows (with all too many gaps) the results in ten patients who had already received varying amounts of atoxyl, soamin, tartar emetic or stibenyl or combinations thereof, without any great or permanent benefit. Table III. is a record of six cases treated first by the intrathecal serum method, lately advocated by Marshall and Vassalo in Uganda. All these cases relapsed, and the effects of tryparsamide on the four who survived is shown.

TABLE I.

Cases Having Received no Previous Treatment.

Case No.	Name	Sex and Age	Course (grammes)	Duration (weeks)	Cell Content of Cerebrospinal Fluid		Remarks
					Before Treatment	After Treatment	
40	Ekeka	M 20	24 00	7½	Failed	10 cells	
44	Angola	M 20	24 00	7	Not examined	Normal	
45	Likumo	M 18	24 00	9	150 cells Trypanosomes	Normal (10 c c)	Second puncture 9 weeks after end of course
72	Salameme	M 40	24 00	6½	500 cells Trypanosomes	Normal (10 c c)	Second puncture 5 weeks after end of course
73	Yaofolo	M 18	24 00	7	470 cells Trypanosomes	Normal (10 c c)	
113	Yaolongolo	F 35	22 50	7½	420 cells	Normal	
115	Bolonge	M 35	12 00	2	540 cells Trypanosomes	50 cells	Second puncture 6 weeks after end of course
131	Kanyono	F 40	21 00	16	R B Cs Trypanosomes Cells + +	50 cells Pressure +	Very severe reaction after first puncture
136	Bekanda	F 25	20 00	12	520 cells	110 cells Pressure +	
138	Afecha	M 14	10 00	9	420 cells	Normal	
139	Yaongonda	F 30	24 00	7	1300 cells	Normal	
146	Mbalika	M 40	24 00	11	2000 cells	Normal	
150	Wayifete	M 40	20 00	12	760 cells Trypanosomes	100 cells Pressure +	
154	Ambale	M 50	20 00	10	1220 cells	180 cells	Second puncture 17 weeks after end of course
163	Likota	M 40	24 00	7	190 cells	10 cells	
165	Bongında	F 25	18 00	8	420 cells Trypanosomes	80 cells	
"	"	" "	8 00	4	80 cells	Normal	
170	Yaiya	F 40	12 00	16	2000 cells	80 cells Pressure +	Very severe reaction after first puncture
171	Bahatiko	F 18	15 00	11½	220 cells	Normal	
173	Lomiya	F 7	10 00	11	Not examined	Not examined	Remarkable clinical improvement.
174	Selenga	F 20	16 00	8	320 cells	Normal	
199	Bokuwa	M 40	20 00	7½	400 cells	Normal	
203	Bochesa	M 25	15 00	4	1200 cells	110 cells	
208	Kondola	M 30	27 00	8½	1300 cells	Normal	
212	Nyololo	M 30	24 00	7	170 cells	Normal	

BRIEF CLINICAL NOTES ON PREVIOUSLY UNTREATED CASES IN TABLE I.

No 40. Ekeka. M., 20. Spastic, sleepy, quarrelsome.

Treatment.—Weekly intravenous 3 gm doses. Well borne.

Discharged.—Rational, well nourished and active.

Progress.—Eleven months after, white colleague reported that he was at work and in splendid health.

No. 44. Angola. M., 20. Spastic, mental torpor.

Treatment.—Weekly intravenous 3 gm. doses. Well borne.

Discharged.—In excellent condition.

Progress.—Eleven months after, reported to be doing his usual work, and quite fit.

No. 45. Likumo. M., 18. Drowsy and mentally unstable.

Treatment.—Weekly intravenous 3 gm. doses. Well borne.

Discharged.—In excellent condition.

Progress.—Two months later, lumbar puncture normal. Eight months after treatment, seen in good health.

No. 72. Salameme. M., 40. Spastic, marked tremors, very thin.

Treatment.—Weekly intravenous 3 gm. doses Well borne.

Discharged.—Still slightly spastic, but better nourished and in fair physical condition.

Progress.—Five weeks later, lumbar puncture normal. Eleven months after discharge, reported to be in good health and doing his work.

No. 73. Yaofolo. M., 18. Emaciated, sleepy.

Treatment.—Weekly intravenous 3 gm doses. Well borne.

Discharged.—Well nourished and mentally alert.

Progress.—Four weeks after, lumbar puncture normal. Nine months after treatment, reported to be in good health and at his work.

No. 113. Yaolongolo. F., 35. Emaciated, mentally unstable.

Treatment.—Weekly intravenous 3 gm. doses. One of 1.5 gm.

Discharged.—In excellent condition.

Progress.—Six months later, apparently in excellent health and refused lumbar puncture "because she was so."

No. 115. Bolonge. M., 35. Sleepy, spastic and quarrelsome.

Treatment.—Bi-weekly intravenous 3 gm. doses. Serious visual impairment after fourth dose.

Discharged.—Almost blind, but physically and mentally very much improved.

Progress.—Improvement well maintained, but sight no better nine months after.

No. 131. Kanyono F, 40. Sleepy, emaciated, very severe reaction after first lumbar puncture, probably due to hæmorrhage from membranes, delayed treatment.

Treatment.—Weekly intravenous 3 gm. doses, but not regularly.

Discharged.—In very good condition.

Progress—Five months later was apparently in excellent health and had put on weight. Refused lumbar puncture.

No. 136. Bekanda F., 25. Sleepy and spastic.

Treatment.—Weekly intravenous 2 gm. doses. Some visual disturbance.

Discharged. Very much improved. For further treatment.

No. 138. Afecha. M., 14. Marked cephalgia, drowsy and thin.

Treatment.—Weekly intravenous 1 gm doses. Well borne.

Discharged—In excellent condition.

Progress—Six months after, reported to be at work in a brickyard and in good health.

No. 139. Yaongonda. F., 30. Very thin and drowsy.

Treatment.—Weekly intravenous 3 gm. doses. Well borne.

Discharged.—Three months' pregnant and in excellent health.

Progress.—Six months after, delivered of a healthy child and apparently quite fit.

No 146. Mbalika. M, 40. Very spastic, unable to walk, covered with sores and sleepy.

Treatment—At first regular 3 gm. doses, later varied on account of visual impairment.

Discharged—Still spastic, but walks well. Mental torpor very slight.

Progress.—Reported to have continued to improve and is still active.

No. 150. Wayifete. M., 40. Spastic, mental torpor, emaciated.

Treatment.—Dosage varied owing to visual changes

Discharged.—Much improved, but visual acuity diminished.

Progress.—For further treatment.

No. 154. Ambale. M., 50. Marked cephalgia, spastic.

Treatment.—Weekly intravenous doses—2 or 3 gm.—rather irregular attendance, slight visual disturbance.

Discharged.—Slight headaches still. General condition good. Treatment to be continued.

No 163. Litoka. M., 40. Spastic, and dull mentally.
Treatment.—Weekly intravenous 3 gm. doses. Well borne.
Discharged.—Condition very good.

No. 165. Bonginda. F., 25. Severe cephalgia, mental instability.
Treatment.—Weekly intravenous 2 gm. doses. Well borne.
Discharged.—In excellent condition.
Progress.—Three months later is still in excellent health.

No. 170. Yaiya. F., 40. Very emaciated and sleepy, very severe reaction after first lumbar puncture.
Treatment.—Varied dosage and irregular owing to visual impairment.
Discharged.—Well nourished and more rational, but still spastic, and with impaired vision. For further treatment.

No 171. Baliatiko. F., 18. Very excitable, tremors marked.
Treatment.—Weekly intravenous doses, varying from 1 to 2 gm., irregular attendance.
Discharged.—In good general condition, but with persistent headache after last lumbar puncture.

No. 173. Lomiya. F., 7. Very sleepy, thin and spastic.
Treatment.—Weekly intramuscular 1 gm. doses
Discharged.—Fat and playful.

No. 174. Selenga. F., 20. Sleepy, emaciated, tremors.
Treatment.—Weekly intravenous 2 gm. doses. Well borne.
Discharged. --Condition excellent

No. 199. Bokuwa. M., 40. Mental torpor, spastic and thin.
Treatment.—Weekly intravenous 3 or 2 gm. doses. Well borne.
Discharged.—In excellent condition.

No. 203. Bochesa M., 25 Severe cephalgia, sleepy and thin.
Treatment.—Weekly intravenous 3 gm doses, until visual impairment contraindicated.
Discharged.—Much improved physically, but sight poor.

No. 208. Kondola. M., 30. Spastic, mentally unstable.
Treatment.—Weekly doses of 3 gm. intravenously. Well borne.
Discharged.—Physical condition very good, mental instability remains.

No. 212. Nyololo. M., 30. Severe cephalgia, sleepy.
Treatment.—Weekly intravenous 3 gm. doses. Well borne.
Discharged.—Condition excellent.

SUMMARY OF RESULTS IN TABLE I.

Case No. 173, Lomiya, a little girl, is included (although I had not the heart to inflict a lumbar puncture on her), for the reason that the clinical improvement in her case was particularly striking. Out of the other twenty-three cases—

> Fourteen showed cell counts of the cerebrospinal fluid within the normal limits in periods varying from one to nine weeks after cessation of treatment. The cell content before treatment in the twelve recorded cases varied from 150 to 2,000 per c.mm., and in four cases trypanosomes were found. Physical and mental improvement was in every case in keeping with these results, and was maintained, so as to give no clinical reason for further treatment for periods varying between one and eleven months. All these patients were able to support a full course without undue delay or irregularity of dosage owing to visual changes, which were absent in all but one, No. 146.
>
> Two cases, Nos. 40 and 163, were discharged after full courses of treatment, well borne, with 10 cells per c.mm., and the former was known to be in excellent health eleven months later, while the latter was but lately discharged, and in very good condition.
>
> One case, No. 131, was discharged with a cell count of 50, and increased pressure, but was still in excellent health five months after. Dosage was irregular, owing to a very severe reaction and subsequent headaches after the first lumbar puncture. The failure to reduce the count completely may be due in part to a reaction after the hæmorrhage, which was evident at the time of puncture. Two cases, Nos. 115 and 203, were unable to receive a full course, owing to serious diminution in visual acuity in the first case, in which two injections a week of 3 grammes had been employed for two weeks, and to less serious trouble in the second case, who was receiving the ordinary weekly 3 gramme dose. Both, however, were markedly improved, and the former was observed for nine months.
>
> Four cases, Nos. 136, 150, 154 and 170, like the above, were unable to support regular and full dosage for fear of permanent damage to the optic nerve, and treatment was prolonged. They are all to receive further treatment, if possible.

It is evident from a study of these results that, if the maximum dose of tryparsamide which can be tolerated be given regularly and in sufficient quantity to patients who are in even the last stages of the disease, there is every chance of reducing the cell count of the cerebrospinal fluid to normal, and of removing, at any rate for a time, all symptoms with the exception of those due to the permanent damage sustained by the central nervous or other systems

TABLE II

Cases Having Received Previous Treatment with Various Other Drugs

Case No	Name	Sex and Age	Course (grammes)		Duration (weeks)	Cell Content of Cerebrospinal Fluid		
						Before Treatment	After First Treatment	After Tryparsamide
2	Oitele	F 14	Atox	10 6				
			Emet	0 45	30	Not examined	250 cells	Not examined
"	"	" "	Tryparsam	8 00	4			Very marked improvement clinically
14	Like	F 25	Atox	2 00				Course not complete
			Emet	0 025	1	Not examined	450 cells	Very marked clinical improvement
"	"	" "	Tryparsam Course not complete					
18	Mama	F 10	Atox	2 7				
			Soam	2 9	42	Not examined	Not examined	Not examined
			Emet	0 15				Marked improvement clinically.
"	"	" "	Tryparsam	15 00	22			
20	Bolamba	M 30	Atox	9 2	12	Not examined	300 cells	60 cells
"	"	" "	Tryparsam	24 00	?			
21	Itilokoka	F 25	Atox	8 5	14	Not examined	150 cells Tryps	R B Cs
"	"	" "	Tryparsam	24 00	11½			Estimated cells normal
26	Rasur	F 20	Atox	5 1	11½	Not examined	1000 cells	Failed
"	"	" "	Tryparsam	24 00	12			Condition very good
31	Besaiko	F 30	Atox	2 0	2	Not examined	Not examined	Normal
"	"	" "	Tryparsam	14 00	8			
34	Vatumbo	M 30	Atox	10 8	14	Not examined	780 cells	50 cells
"	"	" "	Tryparsam	22 00	21			
132	Likunda	F 16	Soam	8 4				50 cells
			Emet	0 9	15	Not examined	50 cells	
"	"	" "	Tryparsam	12 00	9			
154	Bondombe	M 35	Stibenyl	2 1	2	200 cells	150 cells	Normal
"	"	" "	Tryparsam	20 00	10½			

BRIEF CLINICAL NOTES ON PREVIOUSLY TREATED CASES IN TABLE II.

No. 2. Oitele F., 14. Previous treatment had left her with slight visual impairment, with cephalgia and emaciation.
Treatment.—Weekly intravenous 2 gm. doses. Well borne.
Discharged.—Fat and happy, and making good progress in school.
Progress.—Eleven months after, condition maintained, growing well.

No. 14. Like. F., 25. Previous and insufficient treatment left her with severe cephalgia and emaciated.
Treatment.—Improvement marked and rapid on commencing tryparsamide. (Treatment in progress.)

No. 16. Mama. F., 10. Previous treatment had not alleviated symptoms of lethargy, tremors or mental instability.
Treatment.—Weekly intramuscular 1 gm. doses.
Discharged.—Fat, alert and physically fit.
Progress.—Two months after, condition remained good.

No. 20. Bolamba. M., 30. Two months after previous treatment he was still spastic, lethargic and thin.
Treatment.—Weekly intravenous 3 gm. doses. Well borne.
Discharged.—Very marked improvement, not sleepy.
Progress.—Eleven months later, reported to be in good health and doing his work.

No. 21. Itilokoka. F., 25. Was still sleepy after her course of atoxyl.
Treatment.—Weekly intravenous 3 gm. doses. Well borne.
Discharged.—Very marked improvement, not sleepy.
Progress.—Ten months later, reported to be in good health.

No. 26. Basur F., 20. Returned four months after an insufficient course of atoxyl, thin, spastic and mentally unstable.
Treatment.—Weekly intravenous 3 gm. doses at irregular intervals. Well borne.
Discharged.—Fat, rational and alert.
Progress.—Five months after, condition still good.

No. 31. Bessiko. F., 30. Visual symptoms developed after two 1 gm. doses of atoxyl; spastic and mentally unstable.
Treatment.—Doses between 1 and 2 gm. intravenously, cautiously, at weekly intervals.
Discharged.—Vision no worse, physical condition vastly improved. Still spastic.
Progress.—Twelve months after gave birth to a healthy child.

No. 34. Yatumbo. M., 30. Three months after full atoxyl course returned drowsy and mentally dull, though still well nourished.
Treatment.—2 and 3 gm. doses at irregular intervals.
Discharged.—Alert and fit, but slightly unstable mentally.

No. 152. Likunda. F., 16. Course of atoxyl had very little effect, still poorly nourished and spastic
Treatment.—Irregular injections of 2 gm. intravenously.
Discharged.—Condition very good, fatter and active.

No 154. Bondombe. M., 35. The 2.1 gm. of stibenyl made little difference to his general condition, nor to the blood count, nor to the number of larvæ of *Filaria loa* in the peripheral circulation.
Treatment.—Weekly dose of 2 gm intravenously. Well borne.
Discharged.—In very good condition.

SUMMARY OF RESULTS IN TABLE II.

Information as to the cerebrospinal fluid is unfortunately scanty in this series of cases. They were not cases chosen from the start for special observation, with the exception of No. 154, and, although lumbar punctures were permitted before commencing treatment with tryparsamide in eight out of ten cases, examination after treatment, for one reason and another, only furnishes data in six cases.

It may be said, however, that the clinical improvement was scarcely less striking; especially is this so in the case of No. 31, who undoubtedly had a severe infection of the nervous system, but two months after treatment with tryparsamide showed a normal fluid, and a year after gave birth to a healthy child.

The question of the formation of an arsenic resistant strain of parasite, by prolonged administration of drugs which are not sufficiently toxic to the trypanosomes in the nervous system, is undoubtedly supported by these cases, although tryparsamide is able, apparently, to act in its trypanocidal rôle in spite of this resistance.

It is probable that a good percentage of these cases will relapse, and that will confirm the conclusions drawn from Table I., as to the desirability of rapid and maximum dosage treatment.

As might have been expected, Case 154, who had received 2.1 gramme of stibenyl and no arsenic previously, was discharged after a full course with a normal fluid.

TABLE III.

Cases Previously Treated by Serum Therapy

Case No.	Name	Sex and Age	Course (grammes)	Duration (weeks).	Cell Content of Cerebrospinal Fluid		
					Before Treatment	After Treatment	After Tryparsamide
78	Liasa	F 25	7 c c serum 24 hours after		500 cells Trypa	1400 cells	30 cells
"	"	" "	Atox 1 00 Tryparsam 22 00	10		Pressure	Pressure
83	Bokotambuli	M 15	2 c c serum 24 hours after Atox 0 75		Normal	1150 cells	Course not complete Very marked clinical improvement
"	"	" "	Tryparsam course not complete				
94	Losoi	F 16	5 c c serum 24 hours after Atox 1 00		470 cells	Failed	Improving till she met a violent death
"	"	" "	Tryparsam 6 00				
101	Bolauwanyele	M 18	5 c c serum 24 hours after Atox 1 00		700 cells Trypa	1400 cells	
"	"	" "	Refused				Refused treatment, died
102	Loila	F 30	4 5 c c serum 24 hours after Tryparsam 3 00		100 cells	Not examined	Normal
"	"	" "	Tryparsam 18 00	7			
107	Molasi	F 40	6 c c serum 24 hours after Tryparsam 3 00		700 cells	800 cells	30 cells
"	"	" "	Tryparsam 24 00	12			

BRIEF CLINICAL NOTES ON CASES TREATED BY INTRATHECAL SERUM IN TABLE III

No. 78. Liasa F, 25 Condition obviously aggravated by the intrathecal treatment

Treatment—Commenced ten weeks after, weekly intravenous 2 gm. doses Well borne. Rapid improvement

Discharged.—Fat and strong, and doing her work.

Progress.—Five months later seen in excellent health

No. 83. Bokotambuli M, 15 Was originally an early case, and remained fairly fit for eight or nine months after intrathecal therapy. Re-examined eleven months later and found to have serious involvement of central nervous system

Treatment.—Was receiving 2 gm intravenously per week, and had already rapidly improved, was cheerful and worked hard on the station where he was employed

No. 84. Losoi. F., 16. Two months after treatment was very much worse, but started to improve mentally and physically when she met a violent death at the hands of her villagers for having come for treatment to the white man (*sic*).

No. 101. Bolauwanyele. M., 18. Was made so much worse, that after a second lumbar puncture he flatly refused treatment and died shortly after.

No. 102. Loila. F., 30. Was obviously worse after two months with positive gland puncture, and increased nervous involvement.
Treatment.—Intramuscular doses of 2 or 3 gm., and intravenous. Abscess of thigh muscles soon healed, after evacuation.
Discharged.—With slight visual impairment, but condition very good.
Progress.—Six months later she was in excellent health.

No. 107. Molassi. F., 40. She reported again in six weeks on account of headaches. Was found to be worse.
Treatment.—Weekly dose of 3 gm. intravenously. Well borne.
Discharged.—Apparently quite fit.
Progress.—Five months later, seen in good health.

SUMMARY OF RESULTS IN TABLE III.

These results show that, with the author's technique, this treatment does considerably more harm than good. The clinical improvement on the adoption of tryparsamide was very striking, and the four surviving patients are among those who promise to give the most satisfactory results

SOME OBSERVATIONS ON THE ACTION OF TRYPARSAMIDE.

These results can be interpreted as bringing out the following points which are worthy of note. Firstly, that the action of this drug is superior to that of atoxyl in dealing with human trypanosomiasis. This is what might be expected from a consideration of the dosage which it is safe to administer, Dr. Pearce having given no less than 8 grammes of tryparsamide to a full-sized healthy adult in a single dose, whereas it is very unwise to exceed 1.25 gramme of atoxyl at a single administration. Secondly, that the action of this drug is gradual, and there seems to be a minimum total dosage, which is sufficient to render normal the cerebrospinal fluid, of about 20 grammes in the average-sized and well-nourished patient. Case 165 in Table I. illustrates well the progressive improvement in the cerebrospinal fluid, having been reduced from a content of 420 cells and trypanosomes to 80 cells after the administration of 18 grammes, and later to a normal count after 8 grammes more had been given. This is in keeping with clinical improvement, which, although very marked after the first few doses, continues, as a rule, throughout the whole course

Successful treatment seems to depend on maintaining the maximum safe concentration of the drug in the system for a period of six weeks to two months It seems, therefore, impossible to escape the conclusion that the active trypanolytic constituent of tryparsamide finds its way into the central nervous system I have no new evidence in support of this view of a much discussed problem, except that Dr Pearce tells us that some workers in America, who were treating syphilis with tryparsamide, were able to demonstrate nearly as much arsenic in the cerebrospinal fluid after a few hours as was circulating in the blood From animal experiments, conducted by Dr. Pearce, over-dose with tryparsamide was shown to produce pathological conditions in the central nervous system, special mention being made of meningeal hæmorrhages, increased tension in the cerebrospinal fluid, and definite degeneration in the cells of the choroid plexus, all suggesting the possibility of their penetration by the drug

Adams[9] of Glasgow, reports very similar improvement in the abnormality of the cerebrospinal fluid in tabes by the repeated and long administration of novarsenobenzol, by the intravenous route only, and adduces a certain amount of evidence to prove that there is definite penetration

This point is immaterial, however, in connection with this paper, and I am not qualified to enter seriously into any discussion over the question

GENERAL CONCLUSIONS.

These are based on the intravenous administration of tryparsamide, although there is good reason to believe that the intramuscular route is not less efficacious.

(1) The maximum tolerated dose (which should not exceed 4 grammes per week for the full-sized adult), if given regularly for a

period of about eight weeks, is capable of completely removing trypanosomes from, and rendering within the normal, the cell content of the cerebrospinal fluid of even the most advanced cases.

(2) This change in the cerebrospinal fluid is accompanied *pari passu* by a very marked clinical improvement, which has so far been observed to have been fully maintained for periods not exceeding eleven months, the longest time which has elapsed up to date since treatment.

(3) It is possible to estimate the maximum tolerated weekly dose from a consideration of the clinical condition and cell content of the cerebrospinal fluid of the patient, so that these results can be obtained without danger of producing any appreciable degree of visual disturbance.

(4) Improvement is hardly less marked in cases which have previously resisted treatment by other arsenical drugs.

(5) The intravenous method of administration is superior to that of intrathecal serum therapy.

CHEMOTHERAPEUTICAL CONSIDERATIONS.

I am not acquainted with any literature on this subject, and am indebted to Dr Nierenstein for his kindness in pointing out the probable explanation of the action of tryparsamide

Tryparsamide differs from atoxyl, firstly in the fact that the arsenic is held in pentavalent combination instead of in the less stable trivalent form, and secondly that it contains the glycine group.

Moore, Nierenstein and Todd[7] came to the conclusion that the free NH_2 group was the active trypanocidal radicle in atoxyl and allied compounds, and they called it the "trypanophobe" group.

Now the lack of a free NH_2 group in tryparsamide probably accounts for its lessened toxicity, but as it may be hydrolysed in the organism, this group is set free and exerts its toxic action by combining with the protoplasm of the parasites.

A second consideration is suggested by the fact that tryparsamide contains the glycine group, $NH \cdot CH_2CO$. Now this glycine group was found by Wendelstadt to be a constituent of the only drug which was capable of exerting any action on *T. lewisi* in rats.

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THE TREATMENT OF MAL DE CADERAS WITH TRYPARSAMIDE.*†

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Mal de caderas is the biggest economic problem of the whole vast Paraguay Valley. The disease entered the valley from the north and spread slowly down the Paraguay River. Beginning more than fifty years ago, it extended the area of its ravages down through the great state of Matto Grosso, up to and beyond the Bolivian border, and on down the river to Paraguay and the Argentine. The disease became enzootic and has caused tremendous losses every year. In Matto Grosso alone, thousands of horses die annually from the disease, the animals being lost just at the season when they have become well trained and are most urgently needed in the catching of the wild cattle. Some of the larger ranches lose almost all their horses each year, sometimes 200 or more, and the smaller ranches, though sometimes escaping the plague for a year or two, eventually have their entire herds wiped out by the disease.

The disease is caused by a trypanosome, discovered and described by Elmassian[1] and named *Trypanosoma equinum*. Horses are especially susceptible to this parasite. In all my travels, I found no authentic case in which a horse that had once developed characteristic symptoms of paresis, subsequently recovered. Mules are less susceptible; the disease is more prolonged and cases of recovery are not uncommon. The common laboratory animals are easily infected with *Trypanosoma equinum* and usually the infection terminates fatally. Brazilian scientists believe that the disease is transmitted by one of the biting flies, *Lepidoselaga lepidota*, a tabanid, commonly called "matuca" by the natives. Migone,[2] of Paraguay, noted extensive epizootics among a type of large rodent called the "capivara" along the water courses. He observed that the animals showed a very curious type of paresis and readily demonstrated *Tr. equinum* in the blood of the infected animal.

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* From the Studies of the Instituto de Hygiene, São Paulo, Brazil.

† The work reported in this paper represents an extension of the chemotherapeutic investigations carried out in The Rockefeller Institute for Medical Research. The drug used for the work was supplied by the Institute.

Many different drugs have been used in the treatment of the disease without marked beneficial results. Migone[3] has recently reported encouraging results with Bayer "205," the formula for which has not been given. Ranchmen on the Upper Paraguay, however, have used this drug to some extent but find it quite toxic in effective doses (3 grams intravenously).*

The Rockefeller Institute for Medical Research has been engaged upon a chemotherapeutic investigation of the treatment of trypanosome and spirochete infections for several years, and among the drugs made and studied was one which possessed a marked therapeutic action in experimental trypanosomiasis. This drug, the sodium salt of N-phenylglycineamide-p-arsonic acid, was first made in 1916 by Jacobs and Heidelberger[4] and has since been named tryparsamide. The toxicologic and therapeutic studies were carried out by Brown and Pearce.[5] They treated various species of laboratory animals infected with a number of trypanosomes, as for example, the causal agent of sleeping sickness in human beings, of dourine, of nagana, of surra, and also of *mal de caderas*. The therapeutic effects obtained with tryparsamide in these experimental infections and the results of the toxicologic investigations were such that a trial of the drug in human trypanosomiasis (African sleeping sickness) was desirable. The first employment of the drug was made in 1920 by Pearce[6] of The Rockefeller Institute, in patients suffering from this disease in the western part of the Belgian Congo. The preliminary results obtained were encouraging and at the present time the drug is being used by a number of physicians in Africa. In addition, it has been sent to government officials in South Africa and India for therapeutic trials in nagana and surra of domestic animals.

In the course of the investigations of Brown and Pearce, it was noted that tryparsamide was an efficient therapeutic agent in the infections produced by the trypanosome of *mal de caderas*. In view of these results, the Instituto de Hygiene of São Paulo, Brazil, became interested in the subject and procured some tryparsamide from The Rockefeller Institute. Dr. Nova Gomez,* one of the volunteer staff members of the Instituto, repeated the therapeutic experiments of Brown and Pearce, inoculating rabbits with an Argentine strain of *Tr. equinum*, and after the animals were markedly affected, treating them with tryparsamide. The results obtained were wholly satisfactory in those instances in which the treatment was instituted before the animals became extremely ill and prostrated.

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* Personal communications.

The Brazilian Land and Cattle Company hearing of tryparsamide, kindly invited me to use the drug at their vast cattle ranch at Descalvados, Matto Grosso, on the upper reaches of the Paraguay River, where *mal de caderas* is endemic in its severest form. Facilities for travel across western Brazil are meager and the voyage up the Paraguay River to the Descalvados Ranch is a slow and haphazard process. Thus it was possible to study the effect of single doses of tryparsamide administered to a few cases of the disease at widely different enforced stopping places in the course of the voyage. One of these was at the Miranda Estancia and another at the Port of Corumba.

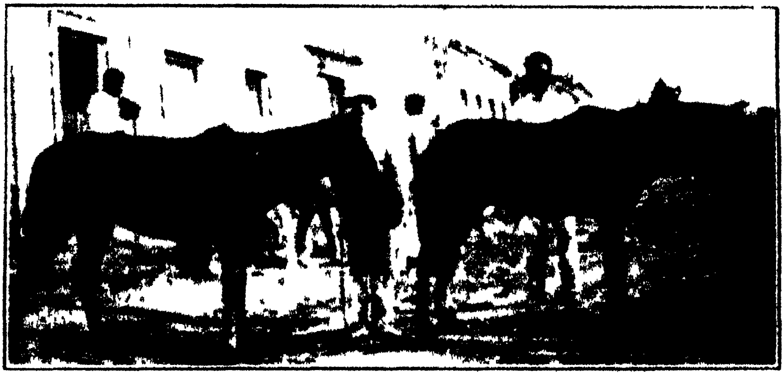

Fig. 1. These horses show the advanced stages of *mal de caderas* with drooping head, sleepy expression, swaying gait, marked anaemia and marked loss of weight. Trypanosomes are very scanty in the blood.

The diagnostic procedure employed to determine the presence of *Tr. equinum* was as follows: 5 cc. of blood were withdrawn from the jugular vein into a centrifuge tube containing a small amount of 2% sodium citrate solution. The blood was centrifuged in a hand centrifuge at high speed for three minutes, when the blood serum became clear and the layer of white corpuscles was clearly defined

Three or four drops of serum were withdrawn with a capillary pipette from the level of the layer of white blood corpuscles, transferred to a slide, covered with a cover-glass and examined immediately for parasites. Two preparations were made from each tube of blood and each specimen was searched for one-half hour before it was discarded as negative. The temperature of the air during the day varied from 90° to 100° F. so that we were working with almost a "warm stage" preparation. The trypanosomes, when present, were easily seen because of their active motility and high refractility and they remained actively motile in the citrated blood for many hours.

Single doses only of 5.0 grams of tryparsamide were administered at the Miranda Estancia and at Corumba. The drug was dissolved in 20.0 cc. of normal saline and administered intravenously.

STUDIES AT MIRANDA ESTANCIA.

Miranda Estancia is at the junction of the Miranda and Paraguay Rivers, in Brazil. This ranch has long been invaded by *mal de caderas*, losing practically all its horses each year, and usually necessitating the annual purchase of 150 new horses. The disease had not commenced at the time of our visit March, 1922. Eighteen of the horses of the home ranch were examined, as our time was limited. All seemed healthy and were actively at work. Thirteen of the eighteen horses examined were considered as negative. Their temperatures (taken per rectum) were normal, ranging from 99.5° to 101° F. and no parasites were found in the circulating blood. Horses Nos. 15 and 17 had temperatures of 102.4° and 102.1° respectively, but blood examination yielded negative results. 10 cc of citrated blood were withdrawn from the vein of each horse and injected intraperitoneally into two young dogs. Six weeks later these dogs were found to be negative.

Horse No. 1 seemed in good condition, but had a temperature of 103° F. and the blood was swarming with trypanosomes, there being ten or fifteen in every microscopic field. This horse was treated at 4 p. m. with 5.0 grams of tryparsamide, given intravenously in 20.0 cc. normal salt solution. At 9 a. m. the following morning, 17 hours after treatment, the blood showed a very few trypanosomes. They were very much less active than those seen the previous day. Many masses of trypanosomes were agglomerated and moving feebly. Phagocytic cells were seen which contained considerable numbers of trypanosomes in all stages of disintegration.

Horse No. 9 had a temperature of 103.9° F. and showed a blood picture similar to that of Horse No. 1, except that fewer parasites were present. The horse was thin but active and at work. He was given 5.0 grams of tryparsamide intravenously at 4 p. m. The following morning at 9 a. m. only one active living trypanosome was found in the blood preparation.

Horse No. 13 was one of eleven survivors that had come through the epizootics of the previous year, in which over 100 horses had been lost. The animal had never been sick and had a normal temperature—100.1° F. Nevertheless, on careful search, a few trypanosomes were found in its blood. This case led us to suspect that the horse itself may be the most important carrier of the disease; the surviving horses which do not develop symptoms and are consequently not shot, carry the parasite in their blood from season to season or from year to year. This horse was treated with 5.0 grams of tryparsamide administered intravenously at 4 p. m. The following morning no parasites were found in the blood.

A subsequent report from this ranch, four months later, stated the *mal de caderas* struck the ranch about two months after my visit, destroying a large number of horses. Horse No. 1 died of the disease, but Horses Nos. 9 and 13 did not develop symptoms.

EXPERIMENTS AT CORUMBA.

While waiting for the up-river launch at Corumba, a port on the Paraguay River, a considerable number of horses and mules were brought to us for examination. A certain proportion of those examined were found to be infected with *Tr. equinum* and may be divided into two groups—early and advanced cases.

Group A. Early Cases.—Eight horses and two mules were in the early stages of the disease. They were active and working daily, and the only symptomatic evidence of *mal de caderas* in six was a gradual loss of weight, despite a good appetite. All had an intermittent or continuous fever of one to three degrees F. but only two were beginning to show the characteristic symptoms of the disease, namely, weakness and a peculiar swaying of the hind quarters. All eight animals were treated with 5-gram doses of tryparsamide, given intravenously.

A report one month later showed that the two mules and four of the horses were active and well, one horse had developed marked symptoms and had been shot, and three horses which had shown some improvement were again on the decline.

FIG. 2 By routine blood examination, three of these horses were found infected with *mal de caderas* The horses were actively at work and the only symptom was an intermittent fever These cases responded well to tryparsamide

Group B. Late Cases. Nineteen horses showed marked symptoms of the disease. They were very thin and weak; some were almost unable to stand, and others were unable to rise after they had fallen Most of them had a high fever. We noted that the horses with high fever usually had the largest numbers of parasites in their blood. In the last stages of the disease the temperature falls to normal or subnormal and only a rare parasite is found in the circulating blood. These cases show marked paresis and at times almost complete paralysis of the hind quarters

All nineteen horses were given 5.0 grams of tryparsamide dissolved in 20 cc. of normal salt solution and injected into the jugular vein. Eighteen hours later those horses that had had the largest number of parasites were re-examined In some instances not a single living parasite was found, though enormous numbers had been seen on the previous day. In three of the cases, living parasites were still found but in greatly diminished numbers. In some instances the blood picture was striking, in that the parasites were agglomerated in great masses of fifteen to thirty organisms forming a slowly revolving, wriggling mass. The phagocytic cells contained masses of parasites in various stages of disintegration.

One month later we received a report of these horses. Eight of the nineteen had died, some of the most advanced cases dying only a day or two after treatment. Eight horses had improved greatly, seemed well and were working hard under trying conditions. Three horses were in about the same condition as before.

These preliminary experiments at Miranda Estancia and at Corumba showed that the administration of a single dose of 5.0 grams of tryparsamide rapidly reduced the number of parasites in the circulating blood and in certain instances appeared to sterilize the blood, as far as could be determined by microscopic examination of centrifuged specimens. In addition there was marked clinical improvement in some animals especially in the early stages of the disease. However, it was clear that a single dose of 5.0 grams was not a sufficient treatment to destroy all the trypanosomes in an infected animal and thus bring about a permanent arrest or a cure of the disease.

EXPERIMENTS AT DESCALVADOS RANCH, PARAGUAY RIVER, BOLIVIAN BORDER.

Conditions at the Descalvados Ranch were most favorable for our field studies. The disease had swept the ranch every year, usually beginning about the end of February. In March, 1922, when we arrived, it had not yet begun to destroy the horses though it was expected daily.

One hundred and thirty-five horses were examined; 18 at the home ranch, 100 at Boa Vista Camp, and 17 at José Alexandrino. None of the horses showed clear-cut symptoms of paresis by means of which the ranchmen could diagnose the disease. It was suspected in some, for they were getting thin despite good care, but none had yet shown the characteristic swaying of the hind quarters. Nevertheless microscopic examination proved that 47 of these horses were infected. Twenty-eight of the group had increased temperatures and a considerable number of parasites in the blood. These we believe were early and active cases of the disease. Some few had no fever, were in good condition and had only a very rare parasite in the blood. These I suspect were latent cases of the disease, for some of them had been through two or three outbreaks without developing symptoms.

Administration of Single Doses.—Two horses that had fever and were becoming thin, and in whose blood trypanosomes were found, were first given 6.0 grams of tryparsamide intravenously in 20.0 cc. normal saline. After 24 hours no parasites were found in the blood. The temperatures of the horses promptly dropped to normal and remained there. Both horses improved rapidly. Seven days later one parasite was found in the blood of Horse D1. It was clear, therefore, that a single dose of 6.0 grams had not sterilized the blood of this infected horse.

One group of 10 horses was given a single dose of 8.0 grams of tryparsamide intravenously. None showed the characteristic last-stage symptom of paresis, though almost all were becoming thin. Six horses had definite fever and showed considerable numbers of parasites in the circulating blood, while four had no fever and only a rare parasite could be found in the blood. All these horses began to improve at once. Their temperatures dropped abruptly to normal, and for 30 days they gained in weight and strength and seemed almost normal, with the exception of Horse A3, which was found dead in the pasture. The owner believed that death was probably caused from snake-bite.

We received a report, about three months later, stating that Horses A6 and A9 had relapsed and were beginning to stagger. A second treatment of 8.0 grams of tryparsamide was then administered to all the horses in this group, nine in number. An immediate improvement followed this treatment and at the last report, four months later, all the horses were well.

These first experiments with tryparsamide showed that single doses of 6.0 to 8.0 grams given intravenously to a 300-kilo horse did not permanently sterilize the blood of the infected animal. On the other hand, the administration of single doses of the drug produced prompt and beneficial effects, such as the cessation of fever and a well marked physical improvement which lasted for several weeks or months. Moreover, the re-treatment with a single dose of 8.0 grams was followed by a normal clinical condition for at least four months. The next step, therefore, in these preliminary therapeutic trials was the administration of repeated doses.

Administration of Repeated Doses.—In order to determine the possible toxic effects of repeated large doses of tryparsamide, a small horse (250 kilos), infected with *mal de caderas*, was given 8.0 grams of tryparsamide intravenously (0.032 gram per kilo). No parasites could be found in the blood 24 hours later. Three days after the first treatment 12.0 grams (0.048 gram per kilo) were given intravenously. The animal showed no toxic or other symptoms whatever, either immediately or later. In this connection mention should be made of the toxicologic experiments of Brown and Pearce.[5] They considered a single dose of 0.75 gram per kilo given intravenously as a lethal dose for the rabbit, since an occasional animal succumbs to this amount. Large, sublethal doses, however, may be repeated at frequent intervals. The approximate lethal dose for the monkey is placed at not less than 1.0–1.25 grams per kilo, administered intravenously, and they cite one instance in which four doses were given in three weeks (0.75, 1.0, 1.25, 1.5). The monkey showed slight signs of intoxication, lasting for a few days, with subsequent recovery. Pearce[6] reports the intravenous administration, with no untoward effects, of three weekly doses of 7.0 grams to a man weighing 62.5 kilos (0.336 gram per kilo). A comparison of these doses indicates that larger doses than 12.0 grams could probably be administered to 300-kilo horses without toxic manifestations.

When the results of this experiment were obtained, 29 infected horses were treated with repeated doses according to the following plan. Twelve horses were given two doses of 8.0 grams of tryparsamide intravenously with an interval of 20 days. Twelve other horses were given two doses of 8.0 grams with an interval of 20 days; the first given intramuscularly and the second intravenously. Five infected horses were held as controls, separated from the treated horses and kept in a distant pasture.

All the horses were about the same weight, approximately 250 to 300 kilos. All were in the early stages of the disease or were latent cases, and showed trypanosomes in the circulating blood in greater or lesser number. Some had considerable fever but the majority had none or a fever of an intermittent type. A few were thin, but none showed the characteristic paresis, and the ranch owner was astonished as one after another of the apparently normal horses was found to be infected.

Thirty-six hours following treatment, all horses were re-examined, and although in every case a careful microscopic search of centrifuged blood was made, no parasites were found. Those horses which had been treated intramuscularly all had marked swelling and tenderness about the area of injection. This edema increased and at the end of 3 days the horses seemed quite ill with considerable fever--103° to 104° F.—and would not eat. The condition entirely disappeared by the end of a week and there was no abscess formation or sloughing. Pearce recommends intramuscular injection with tryparsamide in the treatment of sleeping sickness in human beings, but our experience showed that the intravenous method is preferable in the treatment of horses.

The treated horses were placed in an isolated pasture and left to the care of the ranch manager. Twenty days following the first treatment, the second treatment of 8.0 grams of tryparsamide was administered. None of the horses had developed symptoms, but the controls had all declined perceptibly.

A report received at the end of 3 months stated that *mal de caderas* had broken out in the portion of the ranch herd that had not been examined or treated and some of the horses had died. The five controls had all died. The 24 horses that had received two treatments of 8.0 grams of tryparsamide were working actively and had shown no symptoms of disease.

A second report, 6 months later, and 9 months after treatment, stated that *mal de caderas* had swept the surrounding ranches but that the 24 treated horses had remained well.

DISCUSSION.

In addition to our observations upon the value of tryparsamide in the treatment of *mal de caderas*, certain facts were noted which we believe may have an important bearing upon the epizootology and prophylaxis of the disease

In the first place, the "matuca" which transmits the disease is prevalent during only a short period November to February at Descalvados At the time of our voyage in March and April there were very few to be found along the Paraguay River. It is probable that the fly is not an important factor as a carrier of the parasite, from season to season, but rather acts as a transmitter of the disease during the hot season

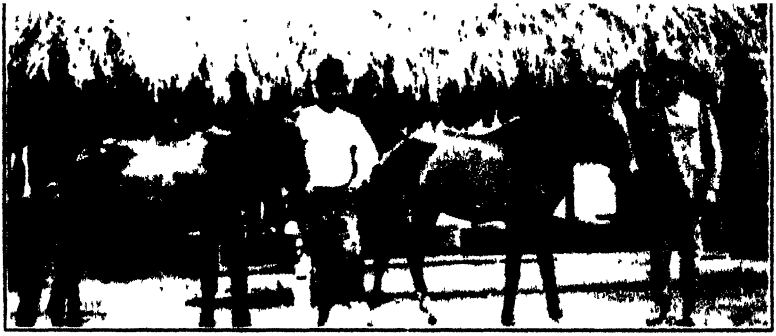

FIG. 3. Note the position of the legs of Horse No 2 His forelegs are wide apart, acting as props, there is a partial paralysis of both hind legs These cases are too far advanced to be cured by treatment with tryparsamide

Migone[2] suggests that the "capivara," a large rodent which is highly susceptible to the parasite, may be an important reservoir of infection. But the "capivara" inhabits the margins of the large rivers, whereas the horses are usually placed several miles from the rivers in upland pastures.

Our observations suggest that the horse itself, and also the mule, which is less susceptible to the parasite, may be the chief latent sources of infection for the herd

To the ranchman, a horse has not been considered as infected with *mal de caderas* until definite and characteristic symptoms of paresis develop; then the animal is shot at once, as it is recognized that a fatal termination is inevitable. Our experiments emphasize the fact that there are two phases of the disease. First, there is the phase of blood invasion. In this stage the horse appears normal and works actively. He may have fever and be losing weight, but the owner can detect none of the characteristic symptoms of the disease. Nevertheless the blood may be swarming with trypanosomes. This is the stage in which there is hope of curing the animal and is also the stage which is of greatest danger to the herd because of the danger of transmission by the fly.

The late stage of the disease with the invasion of the central nervous system and its characteristic symptoms of paresis is the one which is commonly recognized as *mal de caderas*. This phase is not of great importance, either from the point of view of treatment or prophylaxis. Even if the disease be checked, the horse or mule could probably not regain its normal condition. Moreover, these animals are not so great a menace to the herd as they were in the early stages of the disease since the parasites have largely disappeared from the circulating blood.

It is obvious that if the disease is to be brought under control, it must be diagnosed in the early stages. We must recognize that if one waits until one horse in the herd develops paresis, it is more than probable that twenty horses are already in the early and highly infective stages of the disease.

Furthermore it is clear that the disease may be dormant in a herd of horses during several weeks or months when the horses are at pasture during the idle season. When the heavy work begins in the early fall the severe exertion will bring about a rapid decline and in a short time the horse becomes paralyzed and is shot.

In view of these facts together with the results obtained by treatment of the early stages of the disease with tryparsamide, two plans of prophylaxis were proposed for the ranches of the Paraguay Valley, which should prove of practical value in checking the extensive inroads made by *mal de caderas* each year.

PLAN A.

1. Make a microscopic blood examination of all horses between December 1 and January 1 each year. Isolate all positive cases in

a clean, short-grass pasture. (The tabanid is most prevalent in thick scrub.)

2. Take the temperatures of all well horses every two weeks from January 1. All horses showing a temperature of 39° C. (102° F.) per rectum should have another blood examination, and all positive cases isolated and treated. The fortnightly taking of temperatures should continue with the well horses until April 1.

3. Immediately on making the diagnosis, treat all positive cases with 8.0 to 10.0 grams of tryparsamide administered intravenously, repeating in two to three weeks. Three treatments of 8.0 to 10.0 grams should probably be sufficient. Horses under treatment should be isolated in a good pasture and should do no work. All horses, both those that are negative and those that have been treated, may be put together and worked at the beginning of the season, about April 15.

This procedure could be most satisfactorily carried out by a competent veterinarian who would remain on the ranch from January 1 to April 15. It would not be necessary for him to remain at the ranch during other seasons of the year. After one or two years, if found desirable, a personnel of young men might be trained in all the details of the examinations. They could then carry on the work, under competent supervison, at a much reduced cost. The most difficult feature of this plan, however, is the microscopic examination of the blood, which demands the skill of a trained technician.

PLAN B.

In case it is not possible to make microscopic examinations, a less accurate but very practical method of prevention and eradication of the disease is as follows:

1. The rectal temperature of all horses should be taken by December 15. Any horse with a temperature of 39° C. (102° F.) or over should be considered as suspicious; immediate isolation should be instituted and daily temperatures taken for five or six days in succession. If the horse continues with a temperature of from 39° to 40° C., he should be considered as infected, and isolated and treated as in Plan A.

2. All horses with temperatures of less than 39° C. may be considered negative, but should be examined every two weeks, as in Plan A, until about April 1.

This alternative method is not scientifically accurate, but is a rough and ready index of infection with *Tr. equinum* since 99% of all fevers in the horses in Descalvados, between January 1 and April 1, are due to *mal de caderas.* This alternative method would probably not give the satisfactory or the permanent results that would undoubtedly follow the adoption of some such procedure as is suggested in Plan A, but it is suggested because it can be carried out without a specially trained personnel.

In addition to the above methods, directed specifically toward the treatment of infected animals, the following general measures should be carried out:

1. New horses should be brought from cadera-free upland zones. Horses brought from the lowlands may be carriers, and one infected horse introduced into the herd is a potential menace to the whole troop.
2. It is not necessary to carry out active and expensive destructive measures against infected rodents ("capivara").
3. It is highly advisable to keep well horses on high ground in clear, short-grass pastures during the rainy hot season, in order to keep them away from the transmitting biting fly (the "matuca").
4. It is not necessary to waste kerosene and disinfectant in destroying the dead bodies of infected horses, as has been the custom on many ranches. The parasite dies with the horse and the soil does not become infected with the disease.

Since the disease is of a chronic type, it is necessary to continue observations with the drug, tryparsamide, over a period of months or years. This paper is intended simply as a preliminary report in order that interested persons may know of the results of our early experiments.

SUMMARY.

1. Single doses of tryparsamide of from 5.0 to 8.0 grams given intravenously to horses and mules suffering with *mal de caderas* are followed by a marked reduction of the parasites in the circulating

blood. In a number of instances no trypanosomes could be found within 24 hours after treatment. In addition, there was a prompt cessation of fever and a pronounced physical improvement. In general the effect of such a dose lasted from one to three months.

2. The administration of two doses of 8.0 grams, separated by an interval of three weeks, was highly effective. The 24 horses so treated remained well and active for nine months while the five untreated controls had all died during the first three months.

3. No toxic symptoms or other evidence of constitutional injury were observed to follow the administration of tryparsamide. On the contrary, pronounced general physical improvement was the rule.

4. The treatment of animals in late stages of the disease offers only a problematical measure of success, because of the marked involvement of the spinal cord, as evidenced by paralysis and also because of the poor physical condition of the animals.

5. It is suggested that the horse and mule may be the most important carriers of the disease and may serve as reservoirs of the infection from season to season.

6. Two plans of procedure for the treatment and possible eventual eradication of the disease in a herd are suggested.

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FACTORS CONCERNED IN THE PRODUCTION OF LESIONS OF THE EYE IN EXPERIMENTAL SYPHILIS.*

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Syphilis is a systemic disease due to a specific infection. It is characterized by an orderly development of lesions in various parts of the body and by more or less profound constitutional symptoms. It is now known that the infection becomes generalized almost from the time of entrance of spirochetes, still, neither symptoms nor lesions appear immediately. Moreover, if the infection progresses without interference, or if insufficient treatment is given, there are periods during which all outward manifestations of disease disappear and eventually a condition of so-called latency may be established, in which the disease is not apparent, and it is difficult to obtain any conclusive evidence of the continued existence of the infection.

In like manner, it is known that individuals who acquire their infection before birth may harbor spirochetes for years with little or no evidence of disease, and it is equally certain that infection may exist from birth or even be acquired in later life without necessarily giving rise to any characteristic clinical manifestations of syphilis, either local or general.

Viewed from another standpoint, it may be said that syphilis tends to pursue a definite course involving one system of organs after another and theoretically, if the conditions were the same in all cases, there would be no deviation from this course. The results of infection do vary, however, and it may be assumed that any deviation from the established course of events is referable to some cause.

It is not until the existence of such conditions as these is realized that the necessity for a clearer understanding of the biology of syphilitic infections is appreciated. It is extremely difficult to study cause and effect in human syphilis and hence it is often difficult to arrive at any conclusion as to conditions which favor or prevent the occurrence of one form of disease or another. However, many of these problems can be studied to advantage in laboratory animals. This is especially true of certain lesions of the eyes, such as keratitis and iritis, that are of relatively frequent occurrence in rabbits inoculated with *Treponema pallidum*.

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From a study of the experimental infection in these animals, it has been found that involvement of the eyes occupies a clearly defined position in the evolution of disease; that there are certain conditions that favor the development of eye lesions and others that tend to prevent their occurrence. Hence, in discussing factors that are concerned in the occurrence of these lesions, it is necessary that we have a general understanding of the biology of syphilitic infections.

The prime factors concerned in shaping the course of syphilitic infections are three in number. The first to be considered is the causative agent of the disease the ability of the organism to grow and to resist the destructive agencies of the body, its affinity for or adaptation to growth in given tissues, its ability to reach given locations in a viable condition, and finally the ability of the organism to inflict injury whether by direct action at the place of growth or by indirect action through the tax imposed upon the defensive powers of the host.

The second group of factors concerns the resistance of the host. Here we have to distinguish between threshold resistance and actual individual animal capacity The one has to do with the initial result of implantation, the other with the eventual outcome of the infection. In either case, one has to consider individual tissues and the organism as a whole, including adaptation to the growth of spirochetes, susceptibility to injury, the sensitiveness of response, and the ability to react and to withstand injury.

Finally, it should be recognized that the circumstances under which infection occurs or develops are in themselves of great moment. The number of infecting organisms, the vitality of the organism at the time of implantation, the portal and the mode of entry, and such apparently remote influences as climatic or meteorological conditions have all been shown to play important parts in determining the course of infection.

No fixed value can be assigned to any of these factors. Some of them tend to increase the severity of disease or predispose to the development of certain types of lesions, while others neutralize these effects or tend to reduce all manifestations of disease to a minimum. It is perfectly obvious that a given result might be brought about in a variety of ways, hence, any attempt to establish causal relationships without due regard for all such elements as those enumerated is almost certain to lead to confusion. In fact, unless one is extremely cautious, he is apt to magnify the importance of that particular factor or group of factors to which he has given the greatest consideration.

In the case of syphilis, more attention has been paid to the spirochete during recent years than to the host or to conditions that exercise their influence through the host. Thus, there has been a strong tendency to attribute clinical variations in the course of the disease to differences in the biological properties of spirochetes rather than to differences in animal constitution or to the circumstances under which the infection was contracted.

Nevertheless, the focusing of attention upon the causative agent of syphilis has been productive of much good. It has led to a critical analysis of this factor in the equation and, in time, it will be possible to evaluate its importance in relation to others.

We cannot enter into a detailed discussion of this phase of our subject, but it may be recalled that the conception of so-called "strains" of syphilitic virus is founded upon clinical observation and antedates any knowledge of the causative agent of the disease. For the most part, the discussion of this subject has centered about syphilis of the central nervous system but the conception is equally applicable to disease of the eyes. In fact, the first experimental work was carried out in connection with lesions of the eyes and no stronger evidence in support of the conception of selective affinity has been submitted than that first advanced by Nichols and Greene and by Reasoner based upon the production of lesions of the eye grounds.

It has been shown experimentally that the eye is highly susceptible to infection with *Treponema pallidum*. Lesions are readily produced by direct inoculation and are of relatively frequent occurrence as manifestations of a generalized infection. Moreover, the liability of the eye to involvement is greater than that of most tissues since it is not protected to an equal extent by the general reaction that takes place in other parts of the body.

On the other hand, there is no doubt that the particular spirochete concerned in certain infections may be regarded as a predisposing factor but why this tendency to the production of eye lesions should exist, it is difficult to say. Almost any recently isolated strain of *Treponema pallidum* tends to produce a considerable number of eye lesions, chiefly keratitis. However, it is certain that some organisms are more prone to the production of lesions of this class than are others and that there may be distinct differences in the character and severity of the lesions produced by organisms derived from various sources.

Nevertheless, none of these properties appears to be attributable to fixed biological characteristics. With continued passage, the incidence and severity of eye lesions may increase or diminish and both the character of the lesion and the time of its occurrence may be affected. Finally, with a given strain of pallidum, a change in one direction may be followed by a change in the opposite direction. The occurrence of such variations would suggest that the tendency displayed by certain organisms to the production of particular types of lesions represented an expression of an existing state of balance between organism and host rather than an inherent predilection on the part of the organism.

This interpretation is supported by the fact that we have been able to show that all of the pathogenic properties of a given strain of *Treponema pallidum* can be modified to an extreme degree by merely varying the conditions of passage. For example, beginning with a strain of pallidum capable of producing a severe generalized disease (Nichol's Strain V), one line of transfers was maintained by making inoculations from animals with latent infections or from inactive or regressing lesions, while the regular stock transfers of the same strain were made from early or active lesions. At the end of two years, the pathogenic properties of these two substances were compared. The stock strain, having been passed under conditions which tended to give it an advantage over the animal, was still highly virulent and produced a severe generalized infection in a majority of the animals; while the other substrain, having been transferred under conditions which placed it at a constant disadvantage, had lost a great part of its virulence. The primary lesions were comparatively small and of short duration and none of the animals developed generalized lesions other than a metastatic orchitis.

This experiment shows clearly the limitations that surround the doctrine of strains. There is a great deal of evidence to show that under a given set of conditions and at a given time in the life history of an organism, the properties possessed by it may increase or decrease the liability to the occurrence of lesions of given types. However, we are not justified in assuming, that there are distinct strains of pallidum capable of producing entirely different types of disease, unless it be clearly understood that such properties may be lost or acquired by any strain and that in any case the action of such organisms is subject to the operation of outside influences which may completely nullify any existing tendency on the part of such organisms.

Turning to the part played by the host in the evolution of disease, there are a number of facts to be considered. It has been shown that spirochetes multiply more rapidly and lesions are produced more readily by inoculations made in certain locations than in others and that the same conditions apply to the development of secondary foci of infection. At present, it is impossible to say whether this is due to positive or to negative factors. In some instances, the adaptation of the part to the nutritive requirements of the spirochete appears to be of considerable importance, as in the case of the testicle. Again, growth may be determined by the local or general resistance that can be brought to bear upon an infection initiated at a given point and the evidence available would indicate that this is of greater moment than passive nutritive adaptation.

On the other hand, the extent and severity of disease are not necessarily proportional to the rate or extent of multiplication of the spirochetes. On the contrary, it has been shown that efforts to increase the severity of the infection by multiple focal inoculations may be completely offset by an increased reaction on the part of the animal. At the same time, it should be recalled that the reaction of the infected animal is proportional to the demand. For these reasons, an infection that begins insidiously may progress much further than one that assumes more severe proportions during its early stages.

Obviously, the susceptibility of the tissues to injury and the sensitiveness of the animal to such injury have much to do with the reaction that takes place. In its final analysis, therefore, the course of the disease may be determined to a large extent by the sensitiveness of the animal and the promptness and efficiency of the defensive reaction.

These are matters of animal constitution and of animal economy. No two animals react to syphilitic infection in precisely the same manner. In one animal, no characteristic lesion of any kind may be produced, although it can be shown that the animal is infected, while in another animal of the same series, there may be the most extensive lesions and the disease may progress with little or no interruption for months or even years or until it causes the death of the animal. Such extremes are rarely encountered but similar differences are met with in any series of 5 to 10 animals and the proportion of animals with high, low, or intermediate degrees of resistance is relatively constant, being roughly 1:1:3, respectively

Variations in resistance due to sex and to physiological states are equally striking. Females, as a class, are distinctly more resistant to syphilitic infection than males and, as a rule, the pregnant female is more resistant than the non pregnant, although pregnancy, in some instances, has the reverse effect.

Age also has to be considered, but as yet, no data are available for an accurate evaluation of the effect of age upon the course of syphilitic infections.

In the same way and for like reasons, one may obtain types of disease which vary greatly by merely modifying the general conditions under which a syphilitic infection is initiated. An infection produced by spirochetes whose vitality is low will not be the same as one produced by organisms that are highly active. In like manner, an infection produced by intratesticular inoculation tends to pursue a different course from an infection produced by intracutaneous inoculation, or by inoculation of a mucous membrane Again, the character of the disease that one can produce with a given strain of *Treponema pallidum* changes with the season of the year or from year to year. Thus, during the summer months, the disease is always comparatively mild while the periods of greatest severity are spring and fall.

Finally, it is by no means certain that all of the lesions that occur as a result of syphilitic infection owe their origin to a local action of the spirochetes. At any rate, susceptibility to injury undergoes a marked alteration so that the lesions that occur at one stage of the infection usually differ from those of another and the destructiveness of the lesions is in no wise determined by the number of organisms present but by the condition of the host. This change in the character of the local reaction is indicative of a more fundamental alteration in general animal economy and there is considerable evidence to show that other lesions may occur in the course of syphilitic infections which are attributable to a disturbance of animal economy rather than to a direct action of the spirochetes.

From this brief review of factors that play a more or less important part in determining the course of syphilitic infections, it is at once apparent that many conditions may favor or prevent the occurrence of lesions of any given class. We shall not attempt to indicate all of the possibilities that exist in the case of ocular involvement. It is important to bear in mind (1) that lesions of the eyes occur in advanced infections, (2) that the majority of lesions arise from an infection in the episcleral tissues immediately surrounding the cornea, (3) that for the most part the local disturbance is out of all proportion to the severity of the local or general infection and that these lesions are especially prone to relapse, (4) and finally that lesions of the eyes rarely occur in animals that show a prompt and vigorous reaction except in cases where the disease proves to be unusually severe.

From a consideration of these facts, it is obvious that the element of foremost importance in all cases is the reaction to infection. In many instances, involvement of the eyes is due merely to a low grade infection which fails to arouse a full measure of opposition on the part of the animal. In other instances, the situation is entirely different. The infection assumes severe proportions from the beginning and progresses in spite of any effort on the part of the animal to prevent its progress. Under such circumstances, the lesions in the eyes occur relatively early and are frequently of a severe character.

Still, as has been pointed out, certain organisms are more prone to produce lesions of the eyes than are others. Nevertheless, it is doubtful whether this tendency can be attributed to any special affinity for the eye. From what is known of the biology of syphilitic infections, and the variability of such tendencies, it is more likely that manifestations of this kind are referable to some peculiarity in the reaction which these organisms arouse or in their resistance to ordinary processes of control.

Finally, while it is generally assumed that the ocular lesions of generalized syphilis are manifestations of a local infection, it is by no means certain that this is always true. Spirochetes have been demonstrated in some instances but they are usually found in comparatively small numbers and dark field examination of such lesions as those of the cornea not infrequently give negative results. We know of at least one instance in which inoculations made from such a lesion were also negative [*] Moreover, we have observed one case of congenital keratitis and iritis in an animal born of syphilitic parents, in which there was no other evidence of actual infection. There is a distinct possibility, therefore, that disturbances of animal economy may play an important rôle in the occurrence of syphilitic affections of the eyes, either as a predisposing factor or as the actual cause for some of the more obscure alterations that occur in syphilitic subjects.

[*] Dr. Alan M. Chesney and Dr Alan C Woods, Johns Hopkins University, personal communication

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STUDIES BASED ON A MALIGNANT TUMOR OF THE RABBIT.

V. METASTASES.

PART 1. DESCRIPTION OF THE LESIONS WITH ESPECIAL REFERENCE TO THEIR OCCURRENCE AND DISTRIBUTION.

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PLATES 25 TO 29.

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In previous reports (1, 2), dealing with phenomena of tumor growth and the clinical effects observed in tumor-bearing animals, reference was made to the frequent occurrence of metastases following intratesticular transplantation and their absence after other methods of inoculation, and it was pointed out that this was one of the outstanding differences in the results obtained by the use of various methods of transplantation. It was further noted that no constant relationship existed between the rate of growth or the size attained by the primary tumor and the occurrence of metastases in distant organs, but that the development of metastases and the malignancy displayed by the tumor were more closely related to persistence of growth or the ability of the animal to confine the growth to a given area, and hence to the function of animal resistance.

If one may consider metastases from this point of view, a study of the phenomena of metastasis of a given tumor in a large series of animals, should not only throw some light upon those conditions which contribute to or prevent the occurrence of metastases but upon the more fundamental problem of the nature and mode of operation of animal resistance. Such a study has been undertaken, and the results, in as far as they pertain to the occurrence or non-occurrence of metastases, will be reported in this and the two following papers.

On account of the scope of the investigation and the volume of the material used, it will be necessary to divide the report dealing with metastases into three parts. The first two papers will be devoted to a description of the lesions and the circumstances of their occurrence as a further contribution to the picture of the disease produced by this tumor and as a basis for the discussion of factors which influence the occurrence and distribution of metastases. This last phase of the subject will be reserved for Part 3 of the paper.

Methods and Material.

The material upon which this study is based consists of a series of 191 rabbits from the first twenty generations of transplantation in which the phenomena of metastasis were studied in relation to the growth of the primary tumor, the clinical course of the disease, the occurrence of changes in other organs and tissues, and a number of other factors such as the age and breed of the animal, the generation of the transplant, and the season at which the inoculations were made, all of which are known to exercise some influence upon the course of the disease For the most part, the animals were derived from experiments which were conducted primarily for the study of the disease as a whole, and hence from the single point of view of metastases, the material is not so well distributed as might be desired, but all animals have been excluded where any objection might be raised to their use The animals fall into two general groups, first, animals killed at regular intervals of from 1 to 4 weeks after inoculation, and second, animals that died or were killed at various periods during the progress or resolution of the growth or after apparent recovery had taken place, the time ranging from 2 weeks to 7 months from the date of inoculation. For statistical purposes, however, the time limits have been fixed at between 3 and 28 weeks

All animals received a unilateral testicular inoculation, and with two exceptions, no experimental procedure was employed which in itself might influence the course of events The exceptions mentioned included one group of four rabbits which had one testicle removed before inoculation and a group of five rabbits that were inoculated with material that had been subjected to repeated freezing and thawing with a consequent prolongation of 2 to 3 weeks in the period of incubation Otherwise, the variables which figured in the results were those of age, breed, length of survival, and such unavoidable differences as might exist in the quality of material used for the inoculation of different series of animals and the time (season) at which the experiments were carried out

In reporting the results of this investigation, estimates of incidence and distribution will be based upon metastases which could be detected clinically or at autopsy or to gross as distinguished from microscopic lesions In estimating the actual incidence of metastases, all animals showing active, regressing, or healed lesions have been regarded as positive, but in classifying lesions on a time basis, it has been necessary to disregard clinical observations as to the time of occurrence of certain groups of metastases and to eliminate healed lesions, since these cannot be properly correlated with autopsy observations The time given in such classifications unless otherwise indicated is that at which the postmortem examination was made

The method of presentation employed is based upon the animal as the prime factor in determining the course of the disease. Lesions are described from the point of view of the peculiarities of the growth and the reaction which occurs in individual organs and tissues, and the circumstances of their occurrence are analyzed with reference to time and to the character and progress of the disease, the object being to bring phenomena of metastasis into as close relationship as possible with those conditions which determine the course of the disease.

Many of the facts in regard to metastases are represented graphically, and for this purpose, two simple methods of charting have been used, both of which are intended to represent relative rather than absolute values The animals studied are immediately divisible into two main groups, positives and negatives. While we are equally concerned with both groups, our interest is at present centered upon those animals in which metastases occurred. Hence in order to avoid undue distortion of the picture presented, it is necessary to eliminate negative animals and to record the results of incidence of different classes of metastases upon a relative basis

Moreover, the mere presence of metastatic growths may in itself be misleading and the further consideration of the number of foci affected has been introduced in order to give the added idea of the severity of disease as expressed in terms of the incidence and distribution of metastases in any group of animals or tissues. In like manner, the general curve of metastatic involvement has been used in many places as a background for comparison with the curves of individual organs in order to preserve the idea of the relation of metastases in individual organs to the disease as a whole

Incidence and Distribution of Secondary Growths in General.

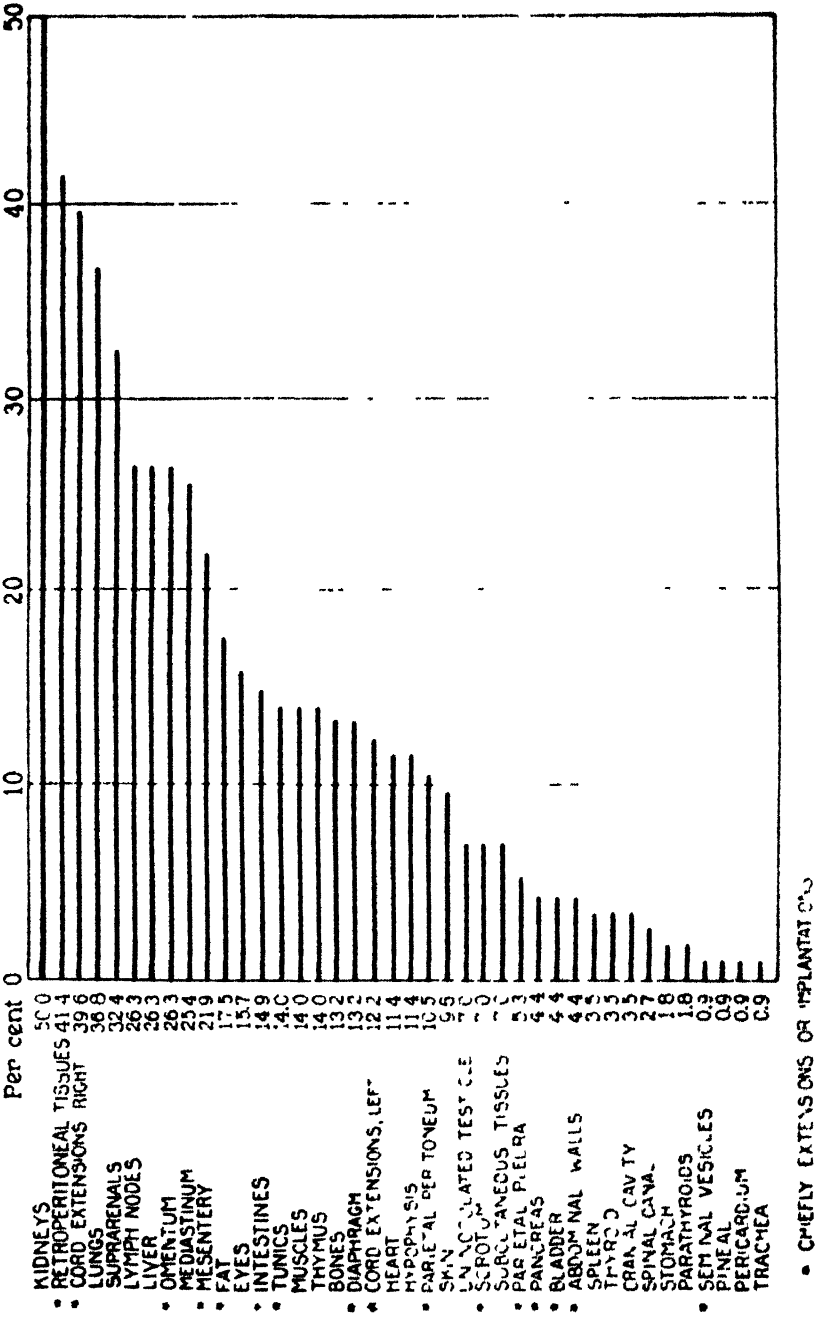
In taking up the subject of secondary growths arising from this tumor, it seems well to emphasize the fact that the tumor under consideration has at all times exhibited a tendency to a diffuse growth with a perivascular arrangement of cells and that invasion of blood vessels and lymphatics has occurred almost from the beginning of the growth or within 7 to 10 days after inoculation. It is obvious, therefore, that some dissemination of cells must have occurred in practically all animals with actively growing primary tumors, and yet, among the animals studied, secondary tumors were found in only 114, while the remaining 77 animals showed no visible extension of the growth beyond the body of the inoculated testicle. It would appear from this, that dissemination of tumor cells does not necessarily imply the development of gross lesions, and this fact is of the foremost importance, since in studying phenomena of metastasis, one is concerned fully as much with the absence of metastases as with their presence.

For convenience, the tumors which developed from a primary growth in the testicle have been divided into three classes, (1) local or regional extensions, (2) implantations on serous surfaces, and (3) metastases to distant parts of the body arising by way of the blood vessels or lymphatics This grouping is intended primarily to indicate degrees of independence and distribution rather than the exact mode of origin, which in reality is one of the problems with which we are concerned.

The mean incidence of secondary growths for the entire group of animals was 60 per cent, and at one time or another metastases occurred in nearly all parts of the body, as shown in Text-fig. 1, which gives the relative frequency of involvement of various organs and tissues in 114 animals. There were great variations, however, in both the incidence and distribution of metastases, and, while it is not intended to take up this phase of the subject in detail, the fact should be noted that at different times and in different series of animals, the incidence of metastases varied between a minimum of about 25 per cent and a maximum of 100 per cent and that the distribution of the lesions was subject to equally marked variations These features of the subject will be discussed when the factors are considered which have influenced the growth and malignancy of the tumor during the course of transplantation

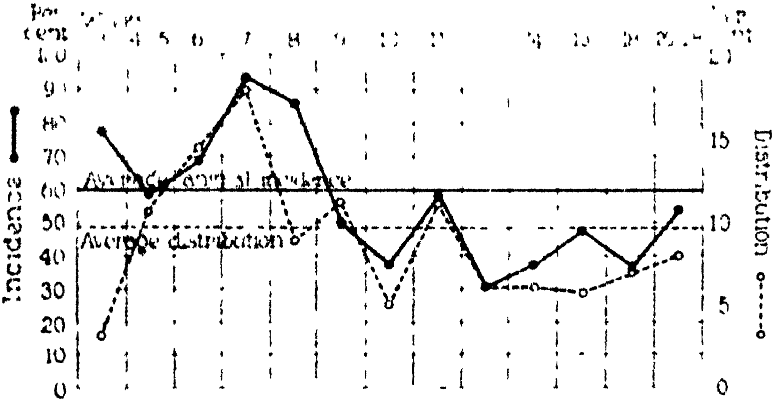
Gross lesions were found as early as 2 weeks after inoculation, and in some instances, actively growing metastases were still present in animals killed as late as 7 months after inoculation. The curve of incidence of all classes of lesions as determined by postmortem examination at periods of from 3 to 28 weeks after inoculation is given in Text fig 2 If this chart is considered with reference to the development of the primary tumor and the subsequent course of the disease in different classes of animals, it possesses a significance which is not otherwise apparent, and since this form of chart will be used with reference to metastases in individual organs, it may be analyzed in some detail

In the first place, two conditions are represented The upper curve indicates the proportion of the animals examined at any time in which secondary growths were found, and may be spoken of as the curve of animal incidence. The lower curve represents the relative number of foci involved as compared with a theoretical possible involvement which was determined by the distribution of metastases in all animals examined thus far, and represents both the extent and distribution of lesions. In order to facilitate comparison of these two curves, the scale of the second curve has been multiplied fivefold.



TEXT-FIG. 1. Relative incidence of secondary tumor growths in different organs and tissues.

A consideration of the conditions found at 3 weeks will show that a great majority of the animals (77 per cent) showed secondary growths but few foci were affected (3.08 per cent). From the 3rd to the 5th weeks, corresponding with the time during which many animals show either an arrest or regression of the primary tumor, fewer animals showed metastases, but those in which metastases were found, showed a decided increase in distribution or the number of organs affected. From this point onward, the two lines proceed almost parallel with a rapid increase in both the incidence and distribution of metastases until the maximum height is reached at the 7th week There is then a marked reduction in both curves extending over a period of several weeks followed by a slight rise with a second drop after which the changes that occur are of minor degree and affect animal incidence rather than the distribution of metastases, but there is a distinct upward trend in the curve of incidence.



TEXT-FIG. 2. Relative incidence and distribution of metastases, plotted with reference to time

These changes are all reflected in the course of the disease The initial drop in the curve of incidence indicates that early metastases may undergo resolution in some animals at the same time that retrogressive changes take place in the primary tumor. The rapid rise in the curves between the 4th and 7th weeks corresponds with a renewal of activity in the primary tumor and the appearance of cases of fulminating malignancy The apparent increase in the curve of incidence is doubtless somewhat exaggerated owing to a preponderance of deaths at this particular time but this is a characteristic feature of the disease. The drop which occurs from the 7th to the 10th weeks is due in part to elimination by death and in part to recovery of a second group of animals The wide discrepancy between incidence and distribution as shown at the 8th and 9th weeks is almost a repetition of the condition at the 3rd and 4th weeks and is doubtless attributable to the same causes. Subsequent elevations in the curve of incidence are attributable to recrudescence of activity and more slowly progressive types of disease which, as may be seen, mature at intervals of approximately 4 weeks. The flattening out of the curve of distribution which occurs during the latter half of the period of observation is of especial importance since, as will be shown later, the maintenance of this particular level is due to the almost constant involvement of one group of organs and of conditions which suggest the development of a selective distribution of cells or an immunity in certain tissues which is not shared by others

The facts which have been brought out show the general tendencies which exist with reference to the occurrence of secondary growths as a whole. It has been found, however, that the conditions which favor the development of metastases are not the same for all organs or tissues, and in order to obtain a clearer conception of the underlying causes for these differences it will be necessary to consider the lesions of different organs individually.

Local or Regional Extensions.

While in many animals, the primary tumor was at all times confined within the testicle, the growth not infrequently invaded the tunics and spread to adjacent tissues or extended outward along the lymphatics and blood vessels either in the form of fairly discrete nodules connected by more or less distinct lines of tumor growth, or as masses continuous with the primary tumor. The resulting lesions, therefore, were of two kinds, but with no sharp line of distinction between them. The picture presented varied in different animals. In some, there were small nodular masses on the outer surface of the tunics, and occasionally the growth involved the scrotum as well. More often there were discrete nodules distributed along the cord, or the growth extended upward into the abdominal cavity in the form of large, irregular masses which not infrequently involved the tissues about the inguinal canal and the lower abdominal and pelvic regions. Examples of the conditions present in cases of this kind may be seen in Figs. 1 to 3, which show varying degrees of the processes described.

These outgrowths from the primary tumor were at times very limited in extent or were confined to the lower abdominal and pelvic regions. As a rule, however, the growth continued upward in the retroperitoneal tissues along the midline of the body or along the outer margins of the lumbar muscles (Figs. 1 to 3). In these cases, the direct connection with the primary tumor was usually lost The growth assumed the form of discrete nodules distributed along the line of the lymphatics with numerous metastases in the retroperitoneal lymph nodes and in the perirenal tissues, and not infrequently, this chain of lesions extended upward in the midline to the base of the neck with the formation of metastases in the retropleural tissues and in the mediastinum.

Growths of the several types described were of frequent occurrence as may be seen by reference to Text-fig 1. Out of a total of 114 animals, there were only 14, or 12.3 per cent, in which the only secondary growths were confined to the tissues immediately surrounding the primary tumor. Extensions up the cord of the inoculated testicle occurred, however, in 39.6 per cent of the animals, and it is important to note that the retroperitoneal tissues were involved with even greater frequency (41 4 per cent), while secondary growths were comparatively rare in either the tunics or the scrotum.

Extension of the primary tumor up the cord and the development of isolated nodules in the epididymis and cord were usually the first clinical signs of malignancy and were frequently recognizable by the end of the 3rd or 4th week after inoculation. In like manner, metastases to the retroperitoneal tissues occurred as early as 2 to 3 weeks after inoculation, or at about the same time and with the same frequency as pulmonary metastases, which shows not only that the cells began to be disseminated at a very early period but presents a striking parallelism in the initiation of metastases by way of the blood vessels and lymphatics.

Lesions of this class rarely gave rise to serious disturbances In several animals, there was obstruction to the right ureter with the production of a hydronephrosis, and in one instance, a growth in the pelvis caused complete obstruction at the neck of the bladder.

Implantations.

Invasion of the tunics and extension of the primary tumor into the lower abdominal cavity were productive of conditions which might be regarded as peculiarly favorable to the development of implantation metastases but lesions of this class were by no means so frequent as might have been expected. In fact, they were less frequent than metastases to the retroperitoneal tissues or to such organs as the lungs and the kidneys, where the distribution of tumor cells was effected either by the lymphatics or the blood stream (Text-fig. 1).

In some instances, there were innumerable masses of tumor growth distributed through all parts of the abdominal cavity, as in Fig. 1, but these cases were comparatively rare. As a rule, there were only a few lesions present, and while some of them were as large as 8 to 10 cm in their greatest diameter, they were usually small and rarely exceeded 1 to 2 cm. in diameter.

These growths occurred most often in the omentum and mesentery but were almost as frequent over the surface of the intestine and diaphragm together with adjacent parts of the abdominal wall. The stomach and bladder were rarely affected and metastases were less frequent on the small intestine than on the cecum and colon. The surface of the spleen was affected in only one animal, and as far as could be determined, there were no implantation metastases on the liver.

In like manner, metastases were less frequent over the lateral and ventral surfaces of the parietal peritoneum than in the pelvic and diaphragmatic pouches, the flanks, or the lumbar regions. Another peculiarity of these lesions was a tendency to localization along the course of blood vessels and lymphatics, as may be seen in Fig. 1, which suggests that in reality some of the lesions classed as implantation metastases may have arisen from cells within blood or lymph vessels. This applies especially to mesenteric metastases

There were a few implantation metastases on the parietal pleura, but again these lesions were rare as compared with the incidence of metastases in the lungs which involved the pleural surfaces. The evidence available, therefore, would indicate that successful implantation of tumor cells on serous surfaces was accomplished with difficulty, and that the hindrance to growth was much greater than that encountered in either the lymphatics or the blood vessels.

Although implantation metastases were of frequent occurrence and occasionally reached a very large size, there was only one instance in which death could be attributed to a growth of this kind. In this animal, an unusually large tumor developed in the mesentery and eventually gave rise to an intestinal infarction.

Metastases in Distant Parts of the Body.

By far the greatest number of secondary tumors, and those of chief importance, were located in parts of the body which were inaccessible to cells from the primary tumor except by passage through lymphatics or blood vessels. Lesions of this class occurred in 100, or 52.3 per cent, of the 191 rabbits studied, while their relative frequency in animals with secondary growths was 87.7 per cent.

Metastases of this class occurred in nearly all parts of the body, as may be seen by reference to Figs. 1 to 4 and Text-fig. 1 which gives the distribution according to incidence in different organs or tissues. The sites of greatest frequency were the kidneys, the lungs, the suprarenals, the lymph nodes, the liver, the eyes, the muscles, the bones, the heart, and the tissues of the mediastinum. In contrast with the high incidence shown by the organs enumerated, it will be noted that metastases occurred very rarely in certain other organs such as the uninoculated testicle, the spleen, and the thyroid, while no metastases were found in such organs as the brain and spinal cord or the salivary glands.

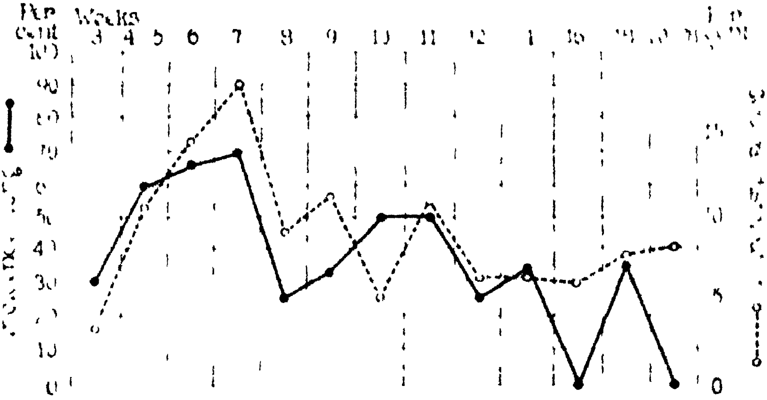
In like manner, the lesions were confined almost entirely to the trunk, the head and neck, and the proximal segments of the extremities; as far as is known, there were no metastases in the ears or tongue or in the feet. In general, it appeared that the frequency of metastases diminished with the distance from the central axis of the body, and this was not entirely attributable to the nature of the tissues composing these parts, since metastases were comparatively frequent in tissues of the same order but nearer the body axis.

On account of the importance of this group of metastases, it will be necessary to consider them in greater detail than those of the two preceding groups. In describing these lesions, emphasis will be placed upon the time relations of metastases in different organs and tissues both with reference to one another and to the course of the disease. The order in which different organs or tissues will be taken up has been determined by the relationships which obtain as expressed in the circumstances under which metastases occurred.

Lungs -Pulmonary metastases were usually small discrete nodules of a grayish white or opalescent appearance; they were usually firm and rarely showed either hemorrhage or necrosis. They were more numerous in the lower than the upper lobes and exhibited a tendency to localize in the pleura (Fig 3) Occasionally, the lungs were thickly studded with these nodules, but as a rule, they were few in number and widely scattered Among animals with metastases, the lungs were affected in 36 8 per cent. The time relations of these lesions with reference to the progress of the disease are especially interesting and can be shown best by means of a chart which records the relative incidence of pulmonary metastases in different groups of animals showing secondary growths, autopsied at from 3 to 28 weeks after inoculation (Text-fig 3)

In general, the chart shows a three period elevation with a decrease in the extent of each succeeding rise which gives the curve a downward slope from the point of greatest elevation between the 4th and 7th weeks These features of the curve may be emphasized, since they are typical of a condition which obtained with a number of organs while in striking contrast with the curves of others

The actual time of occurrence of metastases in the lungs or other organs can be stated only in a general way It may be seen from the relative position of the two curves in Text-fig. 3 that lung metastases occurred ahead of the general involvement of other organs. The earliest pulmonary metastases were noted 2 weeks after inoculation. At the end of the 3rd week, they were more frequent than metastases in any other part of the body, except in the immediate vicinity of the primary growth. Moreover, while there was a reduction in relative frequency during the later periods of observation, pulmonary metastases were still comparatively frequent in all groups of animals up to the 16th week. After this, they were of sporadic occurrence, and the cases observed were without exception among animals of the first few generations in which the disease progressed much more slowly than at present. This fact should be mentioned because an effect of the same kind has been introduced into several other curves, notably those of the kidney and liver. In reality, there is considerable evidence to show that pulmonary metastases were of more frequent occurrence than the actual figures would indicate and that they not infrequently underwent spontaneous resolution without leaving any gross evidence of their previous existence. The features of pulmonary metastases which



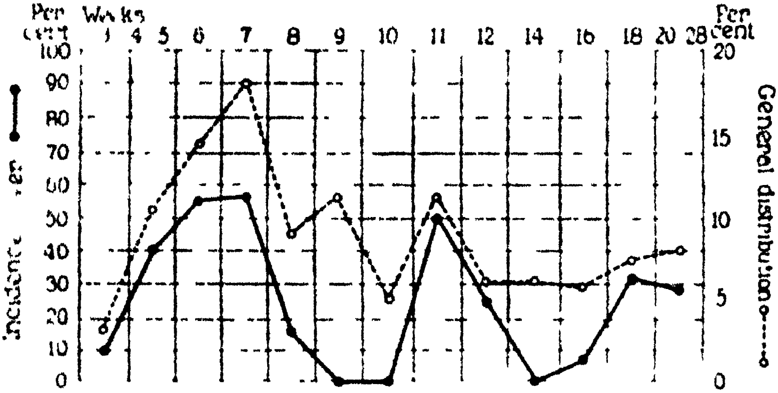
TEXT-FIG 3 Relative incidence of lung metastases as compared with general distribution of secondary growths, at different intervals of time after inoculation.

are of especial interest are their early occurrence and relatively high frequency during the very early stages of the disease, as contrasted with the occurrence of few lesions of small size, and their tendency to diminish in frequency during the later periods of observation After the first few weeks, during which they occurred in all classes of animals, they were in general lesions of severe rather than of mild forms of disease.

Liver.—The growth in the liver varied greatly in different animals. In most instances, there were a few large or medium sized nodules measuring from 0.5 to as much as 2.0 cm. in diameter. Individual lesions were of a soft, medullary consistency and not infrequently showed hemorrhage and necrosis, but they were rarely enclosed by a connective tissue capsule, except in animals with a long standing tumor growth. In a few instances, partially or completely healed lesions were found.

Not infrequently the liver showed a most extensive metastatic involvement with innumerable large or small nodules distributed through all parts of the organ as shown in Figs. 5 and 6. These conditions corresponded with differences in the rapidity with which the disease progressed and from this standpoint are illustrative of similar differences which existed in other organs, especially the kidneys. The growth in these cases was associated with a marked enlargement of the liver. The weight of the organ per kilo of body weight was at times increased as much as two or three times that of the normal animal This change was also proportional to the rapidity with which the disease progressed.

The curve of incidence of liver metastases (Text-fig 4) shows a clearly defined division into three periods, and each period of increase lies within and parallel with the curve of general distribution The notable differences between the two curves are at those points where liver metastases were at their lowest level, indicating that the liver contributed very little to the general picture of the disease at these times The periods of greatest frequency of liver metastases were again periods of widespread systemic involvement The terminal rise might appear to contradict this statement, but the animals responsible for this feature of the curve all came from early generations, as was noted with the lungs



TEXT-FIG. 4. Relative incidence of liver metastases.

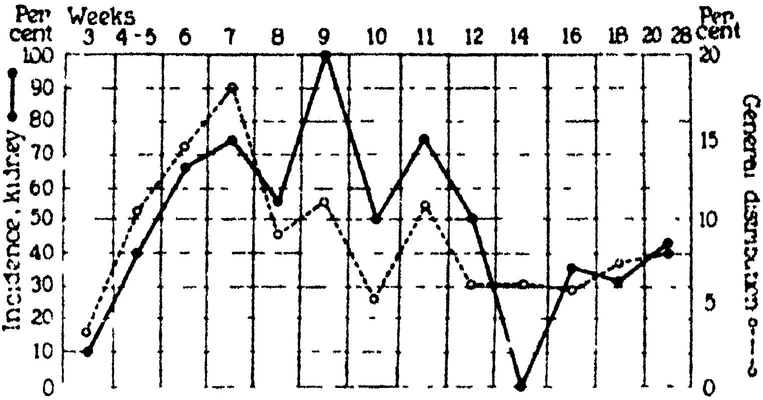
In contrast with pulmonary metastases, those of the liver were much larger, and more numerous, suggesting that opportunities for growth were better than in the lungs but that the organ was in general less accessible to tumor cells and hence was involved only at times when cell distribution was at its optimum.

Kidneys.—The kidneys were not only the most frequent sites of metastases but they also showed the greatest degree of involvement. As a rule, both kidneys were affected, and while in some instances there were only a few large or small nodules, comparable in all respects to those of the liver, the surface of the organ was more often completely obscured by an irregular nodular mass of tumor growth (Figs

2 and 3). Metastases were located primarily in the cortex and in the subcapsular tissues; a few lesions developed in the pelvis of the kidney, but the medulla was practically free, except as it became involved by extension of the growth from other parts of the kidney or from surrounding tissues. As a rule, the growth developed in the outer portions of the cortex and extended outward rather than inward.

In instances of slowly progressive disease the individual lesions were usually large, and not infrequently there was a marked thickening of the capsule. In more rapidly progressing cases, the lesions were smaller but still numerous. These lesions were frequently hemorrhagic in character and gave rise to profuse subcapsular hemorrhages.

Encapsulated, necrotic, and completely healed metastases were found in a considerable number of animals. In these cases, there were usually only a few lesions, but one animal was encountered with almost complete healing of what appeared to have been an extensive involvement of both kidneys.



TEXT FIG. 5. Relative incidence of kidney metastases.

The form of the curve of renal metastases (Text-fig. 5) is different from that of either the lungs or the liver. During the earlier phases, it conforms more nearly to that of general metastatic involvement than does the curve of any other organ. This might be expected on account of the high incidence of renal metastases, but it is important to note that the maximum incidence is not reached until the 9th week, or at a time when metastases in most organs have begun to decline or have already reached a low level. Moreover, a high incidence of renal metastasis was maintained during the first 12 weeks, the curve from the 8th to the 12th weeks exceeding but conforming to that of distribution, indicating that a large proportion of the metastases found during this period was referable to the kidneys. At the 14th week, the curve reaches the base line but again rises with the distribution curve. This final rise, as with the two preceding groups of metastases, was due largely to animals from early generations.

The most significant feature of the curve of renal metastases is its sustained elevation and in this respect it resembles the curve of pulmonary metastasis. Its trend is upward, however, at a time when pulmonary lesions are diminishing, and this difference is even more striking in comparison with the course of events in the liver. These characteristics of the curve, together with the extensive growth of renal metastases, suggest two things: first, accessibility to tumor cells, and second, adaptation to the growth of metastases, or its converse, low resistance to tumor growth.

Heart.—Comparatively few animals showed metastases in the heart. The lesions were located chiefly in the tissues about the coronary sinuses and vessels or were distributed diffusely through the myocardium. In a few instances, masses of tumor cells were found in the right auricle or ventricle, apparently free or attached to the walls of the heart by a slender pedicle.

The metastases comprising this group varied from minute points to nodules measuring as much as 1 cm in diameter. The majority of the metastases in the myocardium and many of those along the course of the coronary vessels were extremely small, semitranslucent masses of a firm consistency and showed no gross evidence of hemorrhage or necrosis, and apparently none of them underwent spontaneous resolution

The larger nodules, on the other hand, were of a medullary character and showed extensive retrogressive changes. Metastases of this type were confined to the epicardial tissues about the base of the heart and apparently represented lesions of a different order from those in the myocardium. In one instance, a lesion of this character was found in the parietal pericardium.

The extent of the cardiac involvement was usually slight, but in a few animals with an unusually malignant disease, there were innumerable foci of tumor growth distributed through all parts of the heart.

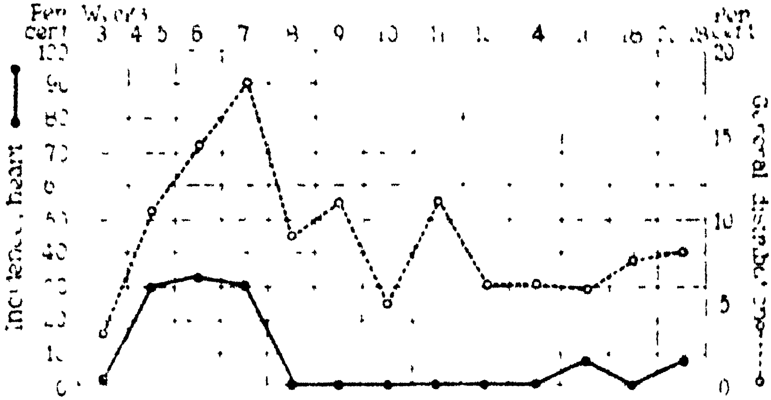
The most significant feature of the occurrence of cardiac metastases was that they were confined almost exclusively to animals with extremely malignant growths This is indicated by the relatively high point reached in the curve of incidence at 4 weeks and the absence of cardiac metastases after the 7th week (Text fig 6). In fact, the cases of most extreme cardiac involvement occurred in animals that showed marked debility 4 weeks after inoculation. The liver shown in Fig. 6 came from one of these animals and metastases were almost as numerous in the myocardium The few exceptions to this rule occurred in animals with marked mediastinal involvement, and the cardiac lesions present were of the same general character as those in the mediastinum and were confined to the epicardial tissues about the base of the heart. A few lesions of this kind were found in animals autopsied several months after inoculation.

Suprarenals.—Metastases in the suprarenals were of frequent occurrence. Of the 114 animals showing metastases, 37 showed metastatic growths in the suprarenals. In 10 instances, both suprarenals were involved, while the right was affected in 12 and the left in 15 animals.

The growth usually arose in the cortex at one or several foci. These tended to coalesce, forming a single mass which occupied the center of the gland and was usually surrounded by a narrow rim of cortex.

The metastases varied in size from a few millimeters to more than 2 cm. in diameter. The growth was of a grayish white or reddish gray color and usually rather translucent The lesions were always soft, and while they were frequently hemorrhagic, they showed comparatively little tendency to mass necrosis.

The mode of growth was peculiar in that it produced a comparatively uniform expansion of the gland with gradual obliteration of both cortex and medulla but showed little or no tendency to invade the surrounding tissues On the other hand as long as the growth was confined to the substance of the gland, there was practically no reaction of any kind about the tumor cells and none in the surrounding tissues When the capsule became involved, however, there was a slight granulomatous reaction but still no encapsulation of the tumor mass such as occurred at times in all other organs with the exception of the hypophysis In like manner, no cases of regressing or healed metastases were found

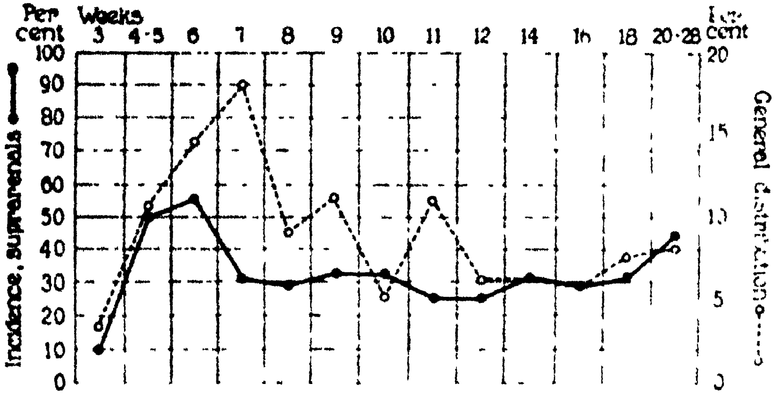


TEXT-FIG 6 Relative incidence of heart metastases

The circumstances associated with the occurrence of suprarenal metastases were also unusual In the first place, they developed early in the course of the disease, the period of maximum incidence being fully a week in advance of that shown by even such organs as the lungs, while a second peculiarity was the maintenance of a practically constant and comparatively high level of incidence from the 7th week onward (Text-fig. 7). Translating these facts into clinical terms, it may be said that suprarenal metastases occurred chiefly in two groups of animals: first, in cases of fulminating malignancy, and second, in animals that showed few or no lesions elsewhere. Some of these had definitely recovered from a more extensive involvement, while others gave no evidence of preexisting metastases in other organs. In brief, when both suprarenals were involved, death occurred, but when only one was involved, there was no serious impairment of vitality on this account, and since suprarenal metastases did not undergo spontaneous resolution, a comparatively high level of incidence was maintained throughout the period of observation. The condition presented by many of these animals was that of an apparent immunity in the presence of an actively growing metastasis in one suprarenal.

Nowhere else was there an exact parallel of the conditions described and they are worthy of especial emphasis on account of their bearing upon phenomena of tumor growth and upon animal resistance.

Eyes.—Eye metastases may be considered to advantage in connection with the suprarenals since they presented some of the same peculiarities that were noted with metastases in those organs and possessed the additional advantage of permitting a correlation between clinical and pathological observations which is helpful in their interpretation



TEXT-FIG 7 Relative incidence of suprarenal metastases.

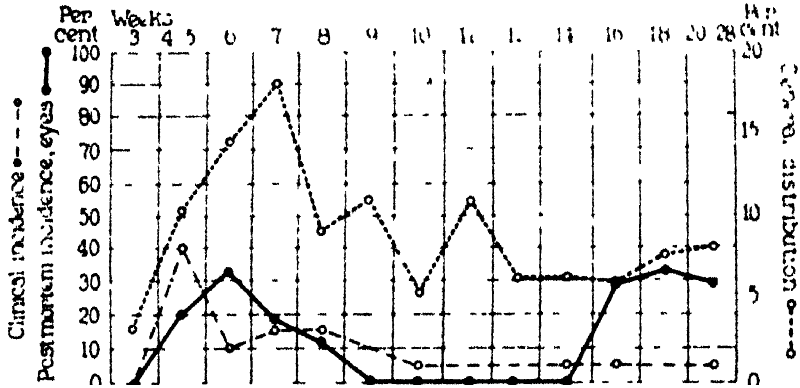
Metastatic growths were noted in the eyes of fourteen animals during life, and a few additional lesions were encountered at autopsy. The right eye alone was involved twelve times, the left twice, while in four animals, metastases developed in both eyes. In one or two instances, the growth was located in the choroid, but, as a rule, it arose from the iris or ciliary body and with few exceptions extended forward through the iris into the anterior chamber

The presence of these metastases was first indicated by a focal or complete pericorneal injection or by the occurrence of hemorrhage into the anterior chamber associated with an irregular prominence in some part of the iris, usually towards the upper or outer margin. The growth of these tumors was very rapid and they presented the same general appearance as primary tumors in this location.

There was one peculiarity of these lesions which may be referred to briefly. In most instances, the cornea remained clear or showed only a slight clouding at the outer margins, and this usually occurred very early and without reference to the extent of the growth. In exceptional instances, however, the entire cornea became very much clouded with an apparent roughening and dryness of the surface which reminded one of the condition described as xerophthalmia. Again, there was no constant relationship between the development of this condition and the extent of the tumor growth. Moreover, the change was not entirely permanent but cleared to some extent in animals in which the tumor regressed.

Eye metastases, as in the case of primary tumors, showed clearly defined periods of progression and regression followed by relapse. In some animals, the growth completely disappeared, leaving only a small fibrous scar, while in others the tumor remained active with no tendency to heal during a period of 3 to 4 months.

As in the case of the suprarenals, eye metastases occurred in two very different classes of animals· first, in animals with highly malignant tumors, and second, in animals with few or no secondary lesions in other parts of the body. These facts are represented in the curve showing the presence of eye lesions at different times as determined by postmortem examination (Text-fig. 8). The curve shows a rapid rise during the 4th to the 6th weeks with a more gradual decline and a second elevation of equal extent during the later periods of observation. This final rise is artificial and is due to the fact that a number of animals with eye metastases were held under observation in order to determine what the outcome in these cases might be. If the existence of these lesions had not been known, this group of animals would probably have been distributed over the periods from the 7th week onward giving a curve not unlike that of the suprarenal metastases.



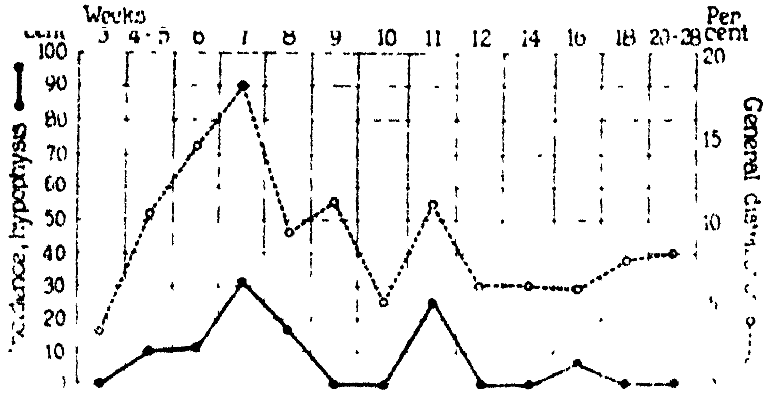
TEXT-FIG. 8 Relative incidence of eye metastases Clinical incidence as compared with postmortem.

The curve of actual incidence (Text-fig. 8) assumes much this form. That is, if the time of clinical record be substituted for the record of postmortem examination, it will be seen that the majority of eye metastases occurred about 4 weeks after inoculation and that there was a slight secondary increase during the 7th and 8th weeks. In brief, 80 per cent of the lesions occurred during the first 8 weeks.

This comparison of clinical and pathological data, when taken in conjunction with the history, is helpful in interpreting the results obtained in other organs where the curve of incidence indicates the operation of a similar group of factors.

Hypophysis.—Metastases in the hypophysis also presented a picture closely related to that of suprarenal metastases both as regards the character of the growth and the conditions under which they occurred.

As far as could be determined, the tumors arose from either the anterior or middle lobe and were identical in character with those in the suprarenals. They were usually small and, with one exception, were confined within the sella which showed a varying degree of enlargement, depending upon the size of the tumor. In one instance, the growth involved both the infundibular stalk and the entire area of the tuber cinereum, forming a mass in the base of the brain which was more than a centimeter in diameter.



Text-Fig. 9 Relative incidence of metastases in the hypophysis

As in the case of suprarenal metastases, there was practically no reaction about the growth in the hypophysis and there was no evidence that these lesions underwent spontaneous resolution.

There were two features of interest in the occurrence of hypophyseal tumors. The curve of incidence (Text-fig 9) brings out in typical form the three waves of diminishing frequency which correspond with similar waves in the curves of many other organs. In fact, this group of lesions was confined almost exclusively to animals with widespread metastases. The exceptions occurred in animals with combined suprarenal and hypophyseal involvement in which few metastases had developed elsewhere or in which preexisting lesions in other organs had healed. In this respect, there was close agreement between the conditions under which metastases occurred in the suprarenals and hypophysis, indicating comparable resistance to tumor growth. There were wide differences, however, in the curves of incidence which may be attributable on the one hand to differences of accessibility to tumor cells and on the other hand, to the fact that hypophyseal involvement almost inevitably caused death.

In the present paper, a description is given of secondary growths arising from direct extension of the primary tumor and from the implantation of cells on serous surfaces, together with metastases in the lungs, the liver, the kidneys, the heart, the suprarenals, the eyes, and the hypophysis. Metastases in other organs and tissues will be described in Part 2 of this paper.

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2. Pearce, L., and Brown, W. H., *J. Exp Med*, 1923, xxxvii, 811.

EXPLANATION OF PLATES.

The illustrations are reproductions of photographs which have not been retouched. Statements of time are from the date of inoculation.

PLATE 25

FIG. 1. 52 days. Physical condition of animal fair; beginning weakness and slight loss of weight, loss of bladder control. Killed. The most striking features of the condition presented by this animal were the extension of the growth upward in the retroperitoneal tissues to the base of the neck and the formation of a profusion of implantation metastases some of which were extremely large and actively growing with little or no necrosis anywhere. The serous cavities were all filled with a blood-stained fluid. There were comparatively few metastases outside the abdominal and thoracic cavities. Note the well nourished condition of the animal, which may be contrasted with Fig. 3. × ½.

PLATE 26

FIG. 2. 49 days. Extreme weakness; moderate loss of weight. Killed. This photograph shows a comparatively small primary tumor in the right testicle which has invaded the tunics and extended along the retroperitoneal lymphatics to the lumbar and perirenal regions. There is a small metastatic nodule in the left testicle, and there are a few lesions in the liver, but the growth is most marked in the kidneys. Many of the lesions show a hemorrhagic tendency, and there is a diffuse subcapsular hemorrhage in the left kidney. There were numerous metastases in other parts of the body. Natural size.

PLATE 27.

FIG. 3. 49 days. Extreme weakness and emaciation. Killed. This animal is intended primarily to illustrate the condition present in fulminating malignancy, although the progress of the disease was not so rapid as is sometimes seen. There were metastases in all the abdominal and thoracic viscera, including the spleen, and in the cerebral meninges, the hypophysis, the eyes, and widespread lesions in the skin and subcutaneous tissues, the deep and superficial lymph nodes, the muscles, and the bones. Many of these metastases are marked by arrows to facilitate identification. This photograph should be examined in connection with Fig. 4 which shows the lesions over the back of the same animal. The lesions of especial interest which may be seen are those in the heart, the skin and subcutaneous tissues, superficial lymph nodes, the muscles (thigh and jaw), the bones of the jaw, and the extensive hemorrhage in the right kidney. The animal also shows a wasting of the fat and of the muscles, especially noticeable in the lumbar region. About ½.

PLATE 28.

FIG. 4. The same animal as in Fig 3. Note the number and size of the cutaneous and subcutaneous metastases and the prominence of the right shoulder, which was due to a growth in the muscle which possibly arose from the scapula. Natural size.

PLATE 29.

FIGS. 5 and 6. The animal whose liver is shown in Fig. 5 died 82 days after inoculation; that from which the liver in Fig. 6 was taken showed an extremely rapid development of skin and bone metastases during the 4th week after inoculation with progressive weakness and emaciation and was killed on the 29th day. Natural size.

The metastases in these two livers illustrate a difference in the character of the lesions seen in cases of rather slowly progressive malignancy and in those that are truly fulminating. The difference in the lesions and the associated change in the organ are typical of the conditions presented by all other organs under similar circumstances and show to what extent the number and character of the lesions present can be taken as an index of malignancy or more properly of animal resistance, since varying degrees of the condition illustrated may be seen in different animals of the same series.

(Pearce and Brown: Malignant tumor of the rabbit. V.)

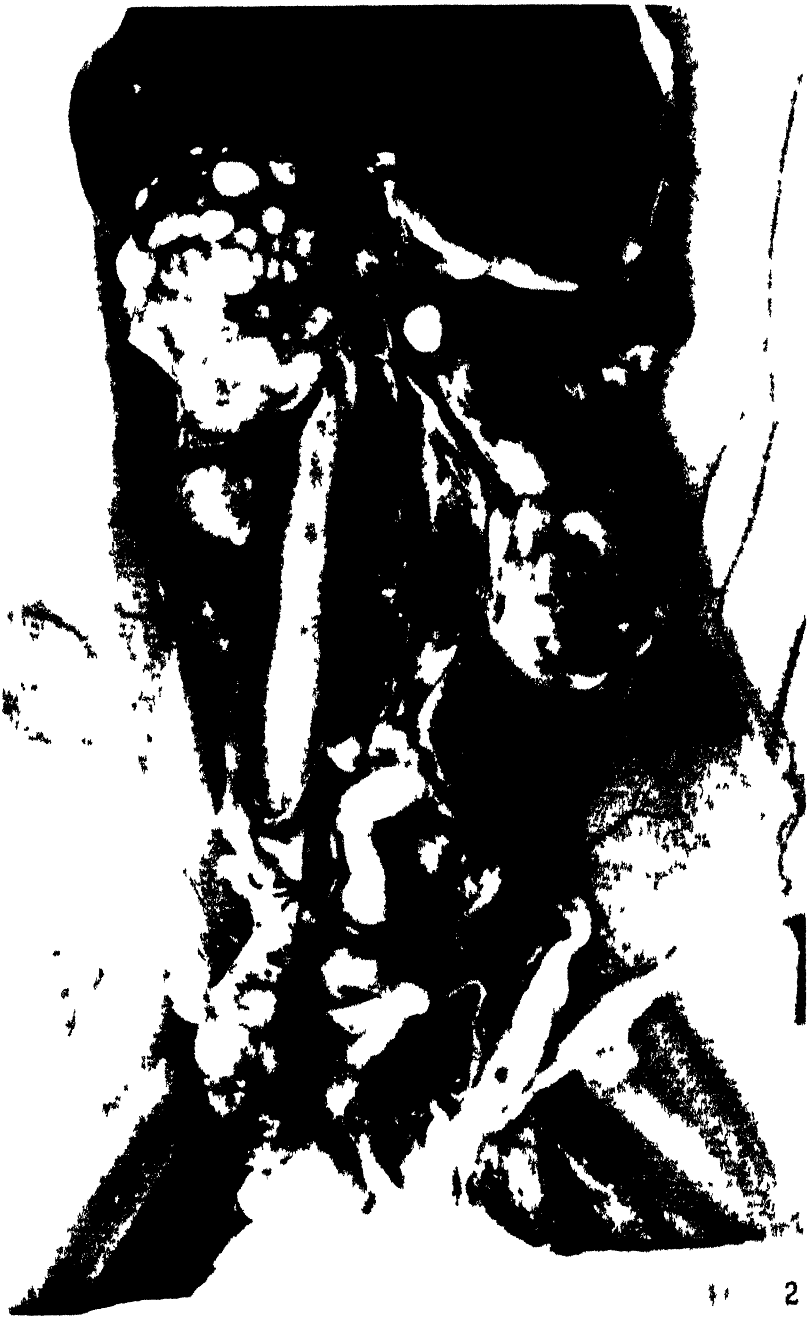

Fig. 2

Pearce and Brown: Malignant tumor of the rabbit. V.

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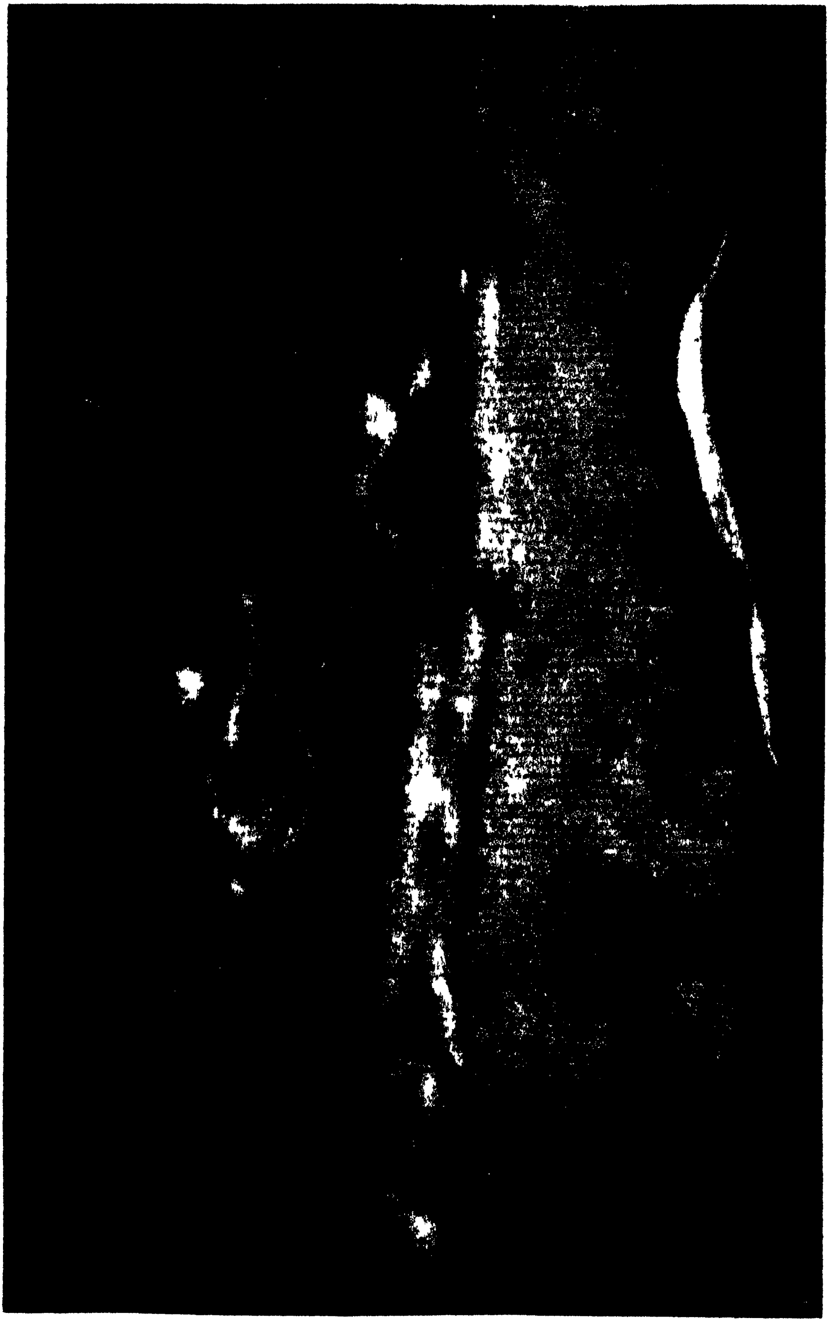

(Pearce and Brown: Malignant tumor of the rabbit. V.)

(Pearce and Brown. Malignant tumor of the rabbit. V.)

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STUDIES BASED ON A MALIGNANT TUMOR OF THE RABBIT.

V. METASTASES.

PART 2. DESCRIPTION OF THE LESIONS WITH ESPECIAL REFERENCE TO THEIR OCCURRENCE AND DISTRIBUTION.

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(Received for publication, May 25, 1923.)

This paper continues the description of metastatic growths contained in Part 1. Consideration of this subject may be resumed with the lesions of the skin and subcutaneous tissues.

Skin and Subcutaneous Tissues.—Comparatively few animals showed metastases in the skin or subcutaneous tissues, but in view of the difficulty experienced in obtaining a growth in these tissues by direct inoculation, the fact that metastases occurred at all is of the greatest significance. With one exception, the lesions were confined to the trunk, the neck, and the proximal segments of the legs.[1] They were most numerous over the anterior part of the body, especially over the back and in the region of the shoulder girdle, and in general followed the lines of blood vessels. In one instance, there were solitary lesions about 1 cm. in diameter symmetrically located on the shins.[2]

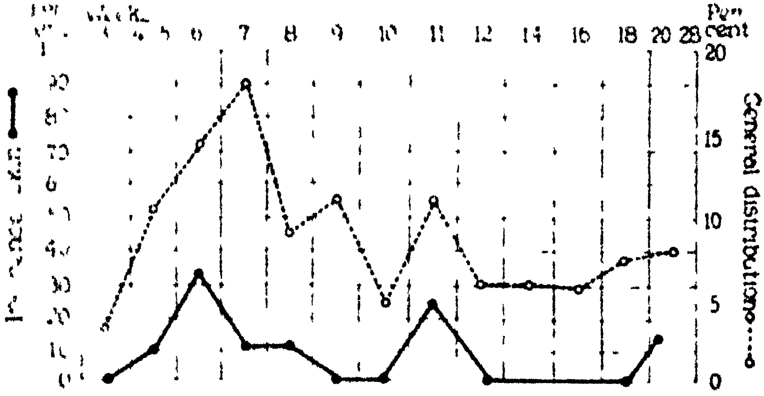
As a rule, cutaneous metastases were small, shotty nodules of an opaque white or translucent appearance, although some of them were of a purplish red color. Occasionally, the lesions measured as much as 1 cm. in diameter, and the larger ones were usually soft and hemorrhagic in character.

The curve of incidence for cutaneous metastases (Text-fig. 1) differed very little from that of the hypophysis with an actual incidence

[1] Part 1, Figs. 3 and 4 (the same animal).

[2] Part 1, Fig. 3.

of metastases comparable to that of the skin (11.4 per cent). The majority of the lesions occurred somewhat earlier. In fact, cutaneous metastases were, as a rule, associated with cases of fulminating malignancy.[1] There was only one instance in which a growth in the skin underwent spontaneous resolution, and there were two animals from early generations in which the disease pursued a chronic but fatal course. Otherwise, the extent of the cutaneous involvement was directly proportional to the severity of the disease and constituted the most reliable clinical index of malignancy. This might have been expected from the structure of the skin and from its known resistance to direct inoculation of the tumor cells.



TEXT-FIG 1 Relative incidence of skin metastases

From the data available, it appears that metastases occurred in the subcutaneous tissues under much the same conditions as in the skin, but on account of the possibility of confusing lesions of this class with metastases in subcutaneous lymph nodes, no attempt has been made to analyze them in detail.

Finally, the development of metastases in the skin and subcutaneous tissues furnishes what is perhaps the most conclusive evidence of the occurrence of marked alteration in animal resistance induced by the growth of the tumor itself.

Muscles.—Metastases occurred in the skeletal muscles with slightly greater frequency than in the skin. They were widely distributed but showed a distinct predilection for the muscles of the thigh, the face, the thorax and shoulder girdle, and the abdominal walls. Other muscles involved were those of the lumbar region, the neck, and the anterior extremities in the order of frequency given. Metastases occurred also in the diaphragm, but in most instances it was impossible to distinguish between implantations and lesions which developed within the muscle itself.

Muscle metastases were usually few in number but were inclined to be rather large (1 to 2 cm. in diameter), soft, and hemorrhagic.[3] They were usually associated with a well marked cellular reaction, but there was little tendency to encapsulation, and as far as is known, they did not undergo spontaneous healing.

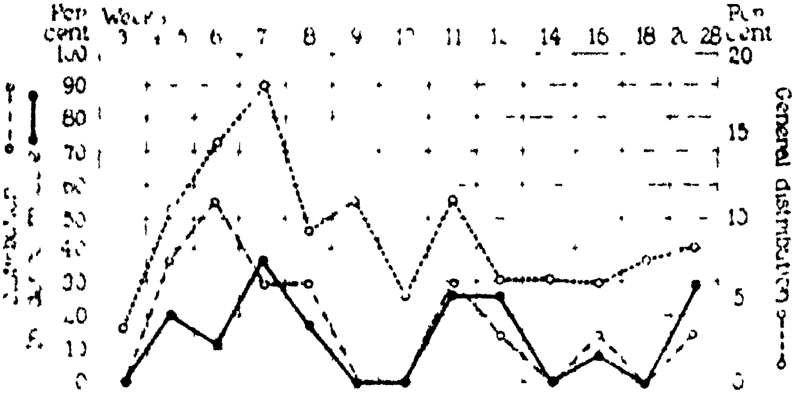
In considering the incidence of muscle metastases with reference to time and the conditions under which they occurred, the factor of distribution, or the number of foci affected, has to be taken into account. As long as one is dealing with single or paired organs, distribution can be disregarded, but in the case of such organs as the muscles, the bones, and the lymph nodes, the number of foci affected becomes a matter of considerable importance.

Muscle metastases were confined to animals with relatively malignant tumors, but occurred with almost equal frequency at three widely separated periods, as shown in Text-fig. 2. That is, from the standpoint of animal incidence, lesions of this class were almost equally divided between slowly progressive and rapidly progressive cases of malignancy. If the curve of muscle metastasis were plotted upon this basis, however, it would be somewhat misleading in that the most extensive muscle involvement was encountered among animals with the most malignant tumors, while there were comparatively few lesions in those with slowly progressive tumors. These facts are brought out by correcting the curve of animal incidence according to the number of foci involved, which places the correct emphasis upon the factor of malignancy and at the same time brings the curve in harmony with those of other metastases whose incidence was determined by a similar group of factors.

Bones.—Bone metastases occurred with about the same frequency as metastases in the muscles or the thymus. In fifteen animals, the lesions were distributed as follows:

Facial bones	12
Calvaria	6
Thorax	4
Posterior extremities	4
Spinal column	3
Anterior extremities	1

There were doubtless other metastases which were overlooked, since the bones could not always be examined with minute care. This list is sufficient, however, to indicate the general tendencies of distribution, the important features of which were the great preponderance of lesions in the axial skeleton and in bones with a comparatively direct blood supply. Moreover, there was a decided preference for cancellous bone, and even in the case of the long bones of the extremities, the growth was more abundant in the epiphyses than in the shaft.

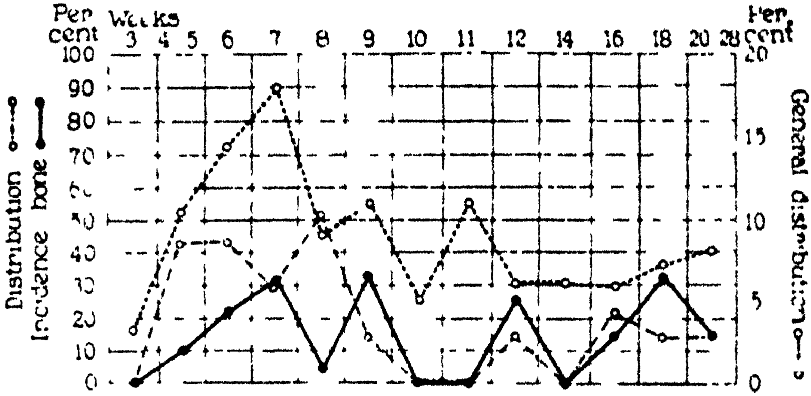


TEXT-FIG 2 Relative incidence and distribution of muscle metastases.

There were a few instances in which metastases appeared to have developed beneath the periosteum in such locations as the mandibles,[1] the distal ends of the ulna, and the external malleoli (not included in the above classification). As a rule, however, the growth arose within the bone, but not infrequently extended outward with the formation of prominent swellings over the surface, and in several instances large fungating masses developed about the teeth. The tumor also showed a tendency to localize in or to extend to the cranial sinuses, and there were two instances in which metastatic growths filled the middle ear on one or both sides.

The bone metastases that were found were rather large, extremely soft, and inclined to be hemorrhagic in character. They produced comparatively little bone destruction, which was probably due to rapid and invasive growth combined with short duration. There was a distinct reduction of bony tissue, however, and spontaneous fractures occurred in a few animals.

No apparent reaction occurred about the tumor mass within the bone, and there was comparatively little about that portion of the growth which extended into the surrounding tissues. In like manner, as far as is known, no bone metastases underwent spontaneous absorption.



TEXT FIG 3 Relative incidence and distribution of bone metastases.

From the standpoint of animal incidence, this group of lesions was again almost equally divided between cases of fulminating malignancy and animals with relatively malignant but slowly progressive tumors. The extent of the involvement, however, was much greater in the first group of animals, as shown in Text-fig. 3. In these respects, there was a close analogy between bone and muscle metastases, and there are points of resemblance between the two curves, but they do not coincide. The chief difference appears to lie in a more uniform distribution of bone metastases among four groups of animals, with a slightly larger proportion of cases occurring during the later periods of observation. In other words, there is evidence of a further shift of incidence from the first to the second half of the scale, while the relative extent of involvement occurring at the two periods is still greatest in animals with rapidly progressive tumors.

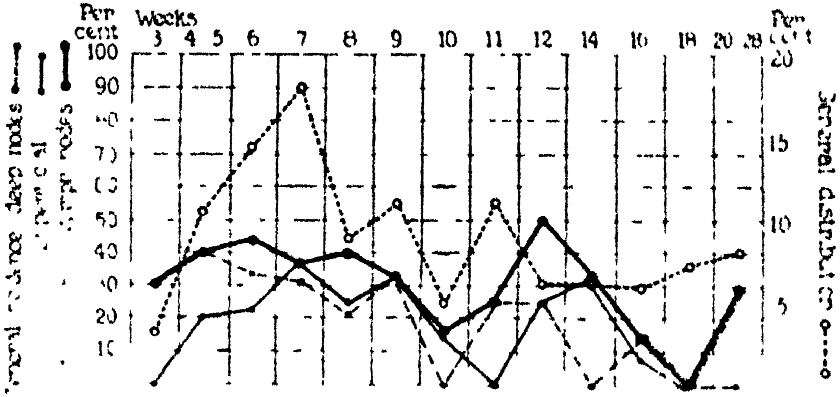
Lymph Nodes - On account of the wide distribution of small masses of lymphoid tissue and the difficulty in determining the exact point of origin of metastases in such places as the subcutaneous tissues, the retroperitoneal tissues, and the mediastinum, it was impossible to observe the same degree of accuracy in recording the occurrence of metastases in lymph nodes that was possible elsewhere. Hence, in order to avoid placing undue emphasis upon this group of metastases, only those lesions were included which could be definitely localized in lymph nodes. When such a distinction could not be made, metastases were recorded as occurring in the tissues of the region concerned. In this way, the actual number of lymph node metastases was undoubtedly underestimated, and this caused a corresponding error of increase in the number of metastases referred to subcutaneous, retroperitoneal, and mediastinal tissues and to the thymus Nevertheless, lymph node metastases ranked high in point of incidence

The distribution and order of frequency of involvement of different groups of nodes were as follows.

Retroperitoneal	18
Mesenteric	10
Posterior cervical	8
Axillary	7
Inguinal	7
Mammary	6
Submaxillary	5
Peritracheal	5
Peribronchial	4
Posterior axillary	3
Anterior cervical	3
Flank	1
Auricular	1

The only groups of nodes of any considerable importance (size) which are not represented in this list are the main mesenteric mass and the nodes immediately connected with it, the deep cervicals, and the popliteals. This is of especial interest when it is recalled that these are the largest nodes in their respective regions. The absence of metastases from the popliteals is not so surprising, since to a very large extent metastases were confined to the head, neck, and trunk, but they did occur in the skin, muscles, and bones of the posterior extremities and even in the loose areolar tissue of the popliteal space. It is more difficult to account for the absence of metastases from the mesenteric nodes and from the deep cervicals, except upon the basis of some structural or functional peculiarity, since all the neighboring nodes and tissues were affected with comparative frequency.

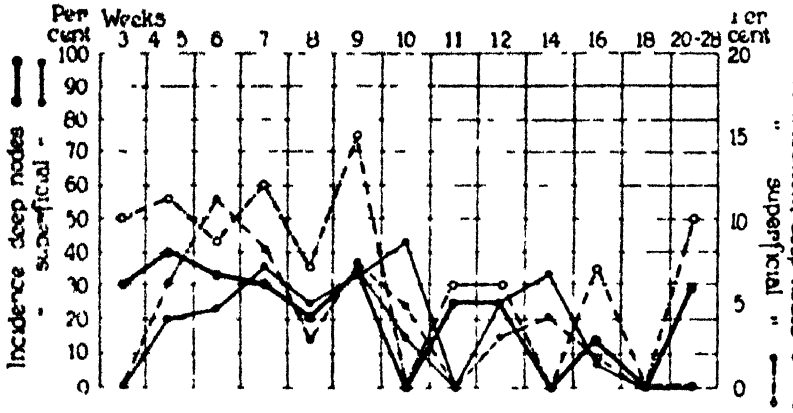
Lymph node metastases were usually rather large[3] and of a grayish pink color. They were soft and frequently hemorrhagic but showed comparatively little necrosis, except in the case of retroperitoneal and mediastinal nodes. These nodes frequently contained large necrotic masses surrounded by a thick fibrous capsule, and it appeared that healing of metastases in the retroperitoneal lymphatics was not infrequent and that it probably occurred in the mediastinal nodes also. In these respects, there was a decided difference between the deep and superficial lymph nodes. In such nodes as the superficial cervicals, the axillaries, etc., there was practically no tissue reaction about the tumor cells, and no regressive changes occurred, except in one animal with a metastasis in the right inguinal node.



TEXT-FIG 4 Relative incidence of lymph node metastases as compared with the incidence of involvement of superficial and of deep nodes

The curve of incidence for lymph node metastases was quite different from that of any group of lesions thus far considered. If all classes of nodes are included, as in Text-fig. 4, there is a high and relatively constant level of incidence from the 3rd to the 9th weeks after inoculation with a second period of increased frequency of even greater extent occurring late in the course of the disease.

If this curve is divided on the basis of deep and superficial nodes (Text-fig. 4), a different picture is presented in that while both groups of nodes show a comparatively high incidence during the earlier periods of observation, those in the drainage area (deep nodes) of the primary tumor show a sustained elevation during the earlier weeks followed by a gradual decline, while the superficial nodes are uninvolved at the beginning but show a gradual rise with some fluctuations and a sharp drop at the 10th week, followed by a second period of increased frequency This curve is not unlike that of bone and muscle metastases but still shows certain distinct differences.

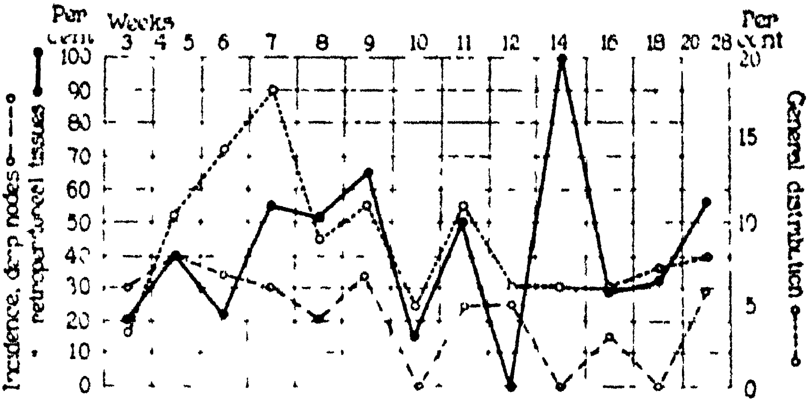


TEXT-FIG. 5. Comparison of the relative incidence and distribution of metastases in deep and in superficial lymph nodes

If the curves for deep and superficial nodes are further modified upon the basis of the extent of involvement, or the number of nodes affected, the differences between the two groups of nodes become still greater, as shown in Text-fig 5. Under these circumstances, the superficial nodes give a curve much like that of the lungs or the liver, showing a maximum involvement, which in this curve really means incidence, at the 6th week with a reduction during later periods, while the maximum involvement of the deep nodes occurs much later and is decidedly more marked during late stages of the disease.

The deep nodes of the abdominal and thoracic groups were involved in all classes of animals without especial reference to the severity of the disease, but in as far as incidence alone is concerned, the involvement was greater in slowly progressive cases than in fulminating cases of malignancy, while with the superficial nodes this condition was reversed. The significance of these relationships will be more apparent when we have considered the incidence of metastases in such locations as the retroperitoneal tissues and the mediastinum

Retroperitoneal Tissues.—As has been intimated, it was impossible to draw a sharp line of distinction between metastases which were distributed along the course of lymphatics in the retroperitoneal tissues and those which were localized in well defined masses of lymphoid tissue. In describing this group of lesions, therefore, the main object is to bring out certain points of similarity and of difference between the two groups of metastases



TEXT-FIG. 6. Comparison of the relative incidence of metastatic growths in the deep lymph nodes and the retroperitoneal tissues

The lesions were alike in all respects and they occurred under much the same circumstances. If the curves of incidence are compared, however (Text-fig. 6), it will be seen that during the first 6 weeks, lymph node metastases were given a higher rating than those in retroperitoneal tissues. This indicates that the earlier lesions, being relatively small and not especially numerous, were more accurately localized than at other times. At the 12th week the same relations obtained and may be attributed to the same causes. On the whole, it appears that the two curves tend to supplement each other. From point to point their direction is usually the same, but occasionally they assume opposite directions, as at the 6th, the 11th, the 12th, and the 14th weeks. Moreover, the curve of lymph node metastases gradually drops to a lower and lower level, while that of retroperitoneal metastases assumes a relatively greater importance in chronic or slowly progressive forms of disease. The significance of the two groups of lesions appears, therefore, to be essentially the same, the difference between them being largely one of degree.

A further relationship to be noted is that between the general distribution of metastatic growths, or the severity of disease, and the occurrence of this group of lesions By comparing these two curves (Text-fig 6), it will be seen that there is a decided lack of agreement, which suggests that the conditions which determine the occurrence of retroperitoneal metastases and metastases in general are not the same and that the conditions which favor the development of retroperitoneal metastases are of relatively greater importance during the late than during the early stages of the disease or are of greater importance in cases of relatively benign tumor growth than in those of fulminating malignancy.

Mediastinal Tissues. - Metastases in the mediastinum presented much the same difficulties of classification as retroperitoneal metastases. In some animals, the growth in the retroperitoneal tissues extended upward into the mediastinum with the formation of tumor nodules along the course of the lymphatics, in the lymph nodes and areolar tissue of the mediastinum, and in the thymus as well [3] In other instances, the lesions present appeared to be independent of any growth in the retroperitoneal tissues

Exclusive of the thymic mass, mediastinal metastases occurred in twenty-nine animals, or in 25.4 per cent of those showing metastases. The lesions were usually few in number but rather large, varying from about 0.5 to 2.0 or 3.0 cm. in diameter, and were either attached to some neighboring structure or were freely movable in the loose areolar tissues.

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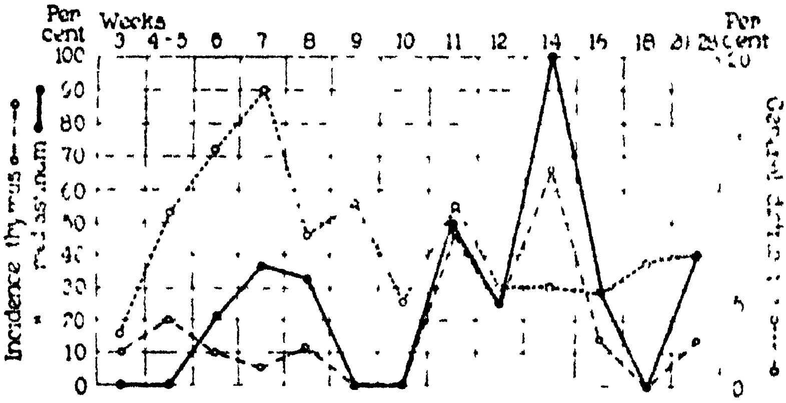
[3] Part I, Figs 1 and 3

The character of the growth also varied in individual animals depending upon the progress of the disease In instances in which the disease was of a rapidly progressive or fulminating character, the growth was composed of a soft, grayish pink mass with a delicate covering of connective tissue, while in animals in which the disease pursued a more chronic course, the lesions were larger and inclined to be necrotic and were usually enclosed in a dense fibrous capsule.

As has been intimated, mediastinal metastases occurred under two entirely different sets of conditions They were of frequent occurrence in cases of high malignancy but were even more frequent in animals with slowly progressive tumors and with comparatively few metastases



Text Fig. 7 Comparison of the relative incidence of secondary growths in the mediastinum and in the thymus

elsewhere except in the retroperitoneal tissues. Represented graphically (Text-fig. 7), there were two periods during which mediastinal metastases occurred, one early and the other late, while the curve as a whole shows an upward rather than a downward trend from the 4th to the 14th weeks.

The curves for the two periods, however, are so different as to suggest that lesions represented by the two divisions may be referable to different causes. Moreover, the first part of the curve closely resembles corresponding parts of a number of the curves previously considered, while the second half is almost a duplication of the corresponding section of the curve of retroperitoneal metastases. The significance of this section is, therefore, quite obvious.

Thymus.—Metastases occurred in the thymic mass with about the same frequency as in the bones and the muscles, but it is uncertain whether any of these lesions could be regarded as having arisen within the substance of the thymus itself. Some of them were definitely located in small masses of lymphoid tissue included within the capsule of the thymus. These lesions were analogous in all respects to metastases in the superficial lymph nodes and occurred under exactly the same conditions; that is, in cases of fulminating malignancy.

In other animals, the thymus was replaced by a tumor mass which virtually filled the superior mediastinum and frequently gave rise to marked respiratory distress. These tumors were surrounded by a dense fibrous capsule and usually showed extensive necrosis but comparatively little absorption of the necrotic material; that is, there was no definite evidence of healing.

In only one instance were metastases found in a thymus that appeared to be normal As in the case of the mediastinum, thymic metastases occurred in two types of disease, the one very acute and the other chronic, but in both instances, there was extreme atrophy of the thymus. Metastases were relatively infrequent among the animals killed within the first 10 weeks after inoculation (Text-fig. 7), while during the later stages of the disease they were almost as frequent as mediastinal metastases, and the curves of incidence for this period virtually coincide.

Perirenal Fat.- Scattered nodules of tumor growth occurred in the perirenal and lumbar fat of a considerable number of animals, and occasionally there was a striking tendency to localization of metastases in the lumbar fat in general, but especially in the perirenal tissues. The conditions under which this occurred are not entirely clear, but the feature of interest was the accumulation of large masses of tumor tissue in the form of multiple nodules which at times completely surrounded the kidneys while the kidneys themselves were unaffected. These animals showed few implantations and few metastases in other organs or tissues.

It appeared that the growth was the result of a rapid distribution of tumor cells by way of the lymphatics, and if this assumption is correct, the absence of renal involvement, or the involvement of other organs, is of especial significance.

Organs and Tissues in Which Metastases Were Infrequent.

In the preceding pages, a description has been given of metastases and the conditions under which they occurred in those organs and tissues which were most often affected. In contrast with these, there were several groups of organs which are of interest on account of the fact that they were rarely or never involved by metastatic growths. These include the gastrointestinal tract, the pancreas, the genitourinary organs, the central nervous system, the spleen, the thyroid and the parathyroids, and the pineal gland.

Gastrointestinal Tract.—There was only one instance of a clearly defined metastasis in the walls of the stomach, and even implantations on the serous surface were comparatively rare. In the one instance recorded, the growth presented the typical appearance of a peptic ulcer. The lesion was about 1 cm. in diameter with a depressed ulcer at the center surrounded by an elevated margin which was composed of tumor cells located chiefly in the submucosa.

The intestine was affected more often and was among the most frequent locations of implantation metastases. As a rule, however, the lesions in the intestine were small and were located either in the subserous or submucous tissues. In two instances, comparatively large tumors developed, and one of these led to an intussusception which caused the death of the animal.

Pancreas.—In view of the frequency of metastases in the omentum and mesentery (implantation), it is remarkable that there were only five instances in which secondary growths occurred in the pancreas or in that portion of the mesentery occupied by the pancreas.

Genitourinary Organs. Omitting the kidneys, the genitourinary organs were rarely involved There were comparatively few implantations on the bladder and only one metastasis of any considerable size in the bladder walls. This was situated in the trigone and formed a nodule about 8 mm. in diameter which projected from the inner surface. The prostate and seminal vesicles were affected only once by a growth which extended downward from the retroperitoneal tissues.

In like manner, metastases to the uninoculated testicle occurred in very few animals, and even extensions and implantations on the cord and tunics of the uninoculated testicle were comparatively rare. In view of the ease with which successful inoculations were obtained in the testicle, the low incidence of metastatic growths in the uninoculated testicle is especially significant as an indication of the relative importance of so called suitability of soil and of other factors in determining the location of secondary growths.

Central Nervous System.—There were five instances in which metastases were found in the meninges of the brain or cord. The origin of these tumors was not certain in all cases. Those in the cranial cavity appeared to have developed in the meninges, while those in the spinal canal arose either in the bones or in the surrounding tissues and entered the canal through the intervertebral foramina.

It is a striking fact that not a single case of brain or cord metastasis occurred. Here again we are confronted by the total absence of metastases from an organ which is highly susceptible to inoculation and in which primary tumors grow with the greatest ease.

Spleen.—Out of a total of 191 animals examined, there were four in which metastases occurred in the spleen as compared with thirty cases of metastasis to organs of a purely lymphoid character. The lesions were small and produced little or no local reaction, and all of them occurred in cases of fulminating malignancy. These findings are probably in accord with general experience, but they are nevertheless significant as an indication of the presence of conditions of some kind which prevent the occurrence of metastases in the spleen.

Thyroid and Parathyroids.—Metastases in the thyroid are of especial interest in comparison with the conditions presented by the suprarenals and the hypophysis as regards both the frequency of occurrence and the character and extent of the local reaction. There were thirty-seven animals with suprarenal metastases and thirteen with metastases in the hypophysis, while there were only two instances recorded in which visible metastases occurred in the external parathyroids and four in the thyroid. In two of these animals, it appeared, however, that the growth might have arisen from an attached or internal parathyroid rather than from the thyroid itself. There was one other animal with a metastasis in the trachea beneath the isthmus of the thyroid, but as far as could be determined, the thyroid itself was not involved.

These growths were comparatively small, the two largest measuring not more than 6 to 8 mm. in diameter, while the others were barely visible to the unaided eye. All of them occurred in animals with extremely malignant tumors, whereas suprarenal and hypophyseal metastases were of frequent occurrence in animals with slowly progressive tumors or with few metastases elsewhere.

The low incidence of gross metastases in the thyroid does not appear to be due to a failure of tumor cells to reach the thyroid. Numerous small collections of cells were found by microscopic examination, but in all instances, they were submerged beneath a massive lymphocytic and fibroblastic reaction which again was in striking contrast with the absence of such reactions in the suprarenals and hypophysis.

When it is recalled that gross metastases occurred in only a few animals with tumors of a most malignant character, indicative of an exceptionally low constitutional resistance, it would appear that the factors of constitutional and local resistance are of especial importance in determining the occurrence of thyroid metastases, and this probably applies to the parathyroids as well.

DISCUSSION.

The description which has been given of the metastases produced by this tumor will serve to convey a general conception of this feature of the disease. It will be seen that here again we are dealing with conditions which in many respects are closely analogous to those that obtain in man but with the added advantage of being able to produce an almost endless series of changes from a given tumor stock and of being able to interrupt the process at any point for the purpose of tracing the successive stages in its development. It is thus possible to link together cause and effect in a way that at best is extremely difficult from the study of human material.

Reviewing the facts presented, one is first impressed by the unusual possibilities of this tumor for the production of metastases, affecting, as they may, nearly all the organs or tissues of the body. Still, it will be seen that there is the greatest diversity in the results obtained in different animals under precisely the same experimental conditions. In the first place, a distinction may be drawn between animals that develop metastases and those that do not, while a further division may be made between animals that apparently recover after metastases have developed and those in which the disease goes steadily onward.

Moreover, among the animals of the last group, the character and distribution of the lesions and the course of the disease vary to such an extent in different animals that, if the two extremes were compared, one could hardly recognize them as manifestations of the same etiologic agent. In one group of animals, metastases develop slowly and are confined largely to the region of the lymphatics which lead upward from the primary tumor to the mediastinum, while in the other, the development of metastases takes place with incredible rapidity and few organs or tissues are spared. In the one instance, the disease is slowly progressive, while in the other, it is fulminating in character. Between these two extremes, one finds various modifications and combinations of the two outstanding types of metastatic involvement.

Finally, by a similar process of analysis, it may be seen that practically the same conditions obtain with reference to the development of metastases in different organs of a given animal or in the homologous organs of different animals.

At first sight, all of these differences may appear to be largely matters of chance but upon closer examination, it will be seen that, in as far as individual organs and tissues are concerned, there is a remarkable consistency in their behavior and that such variations as occur in a given organ or tissue bear a definite relation to the variations which occur in other organs of the same animal. In brief, it will be seen that the peculiarities displayed by individual animals are founded upon constitutional differences which are most clearly expressed in the behavior of the individual elements which form the more complex animal organism.

If animal resistance is considered from this point of view, it is obvious that the most direct method of approach to the problem of resistance to tumor growth is by a careful investigation of conditions which obtain in individual organs and tissues and the relations which exist between one group of organs and another. From investigations of this kind, it may be possible to reconstruct the whole.

This conception is applicable to the study of metastases, since they represent an expression of one form of interaction between tumor and host, and the data bearing upon the subject of metastasis will be taken up from this point of view in the next paper of the series.

SUMMARY AND CONCLUSIONS.

As a part of a general investigation of a malignant tumor of the rabbit, the phenomena of metastasis were studied in a group of 191 animals. The results of this investigation are presented from the standpoint of the relation of metastasis to animal resistance. The incidence and distribution of different classes of lesions are given and the peculiarities of the growth in different organs and tissues are described with especial reference to the time and circumstances of their occurrence and the relation of metastases in one organ or tissue to those of another.

It was found that while the picture presented by different animals varied greatly, there was a remarkable degree of uniformity in the character of the lesions in a given organ or tissue and in the circumstances under which metastases occurred in a given location. It was thus possible to establish a relationship between the distribution of metastases and the function of animal resistance.

In brief, the conclusion was reached that the peculiarities of metastatic involvement displayed by individual animals were not entirely attributable to chance distribution of tumor cells but that they were founded very largely upon constitutional differences and that the picture presented in any given instance represented an expression of the interaction between tumor and host, the character and force of which were indicated by the nature and distribution of the lesions or by the organs and tissues affected.

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STUDIES BASED ON A MALIGNANT TUMOR OF THE RABBIT.

V. METASTASES.

PART 3. FACTORS THAT INFLUENCE OCCURRENCE AND DISTRIBUTION.

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(Received for publication, May 25, 1923.)

In studying phenomena of metastasis of spontaneous tumors, whether in laboratory animals or in man, one has but a single example of the results of interaction between tumor and host, and while it is possible to accumulate large groups of cases composed of tumors of similar or even identical character, one is still confronted by the fact of individuality or a lack of knowledge of how a given tumor might behave in another individual. Our knowledge of phenomena of metastasis is founded largely upon the study of such material, but in spite of the difficulties referred to, it has been possible to show that tumors of a given class tend to metastasize by certain paths and to localize in certain parts of the body. It has also been found that, while the great majority of tumors of a given type show a remarkable degree of conformity in their general behavior, there are striking exceptions to these rules, and that a wide range of possibilities is presented by almost any variety of malignant tumor. Moreover, it is well known that tumors ordinarily regarded as benign may upon occasion assume malignant characteristics and give rise to metastases in distant organs.

In brief, our experience with spontaneous tumors has led to the creation of a conception of essential conformity in the behavior of tumors of a given class and of possible variations which is of inestimable value in dealing with malignant tumors. At the same time, comparatively little is known of the underlying causes for this behavior. No one knows whether these features of tumor growth are attributable to some tendency on the part of the tumor cell or whether after all the conformity in behavior may not be referable to the same causes as are responsible for the development of the tumor. At any rate, the objective evidence of cellular activity is the thing that has been most forcibly impressed upon us by our contact with malignant disease, and it may be that this experience has tended to magnify our conception of the relative importance of the tumor cell as an aggressive agent with selective capacities and to minimize the importance of other factors. In the same way, we have been led to believe that tumors are exempt from the general laws or principles governing pathological action or that they are autonomous and obey no known laws.

As long as our knowledge is confined to facts that may be gained from a study of spontaneous tumors and the single expression of action of a given tumor, it is difficult to escape such conclusions, but our point of view may be greatly enlarged by multiplying instances of the action of a given tumor stock and by varying the conditions of its activity. If, therefore, nothing more could be gained from the study of transplanted tumors, it would still be worth the while to examine into the one problem of the conformity of tumors to the action of principles of any kind with a view to determining the causes which are responsible for the uniformity in the behavior of tumors of a given class and the peculiarities which characterize the behavior of different classes of tumors. In other words, to what extent are the characteristics of tumor growth due to essential attributes of the tumor cells, and how far may these cells be influenced by factors which are within the host or by factors entirely distinct from either but operative through the host rather than directly upon the tumor cells? This is the essence of the problem of metastasis.

In the present instance of a rabbit tumor, the opportunity was afforded of making a careful study of the animal with the spontaneous tumor (1) and since then a long series of observations has been carried out on tumors derived from this parent stock (2-4). The spontaneous tumor metastasized both by way of the lymphatics and the blood stream, the organs affected being the regional lymph nodes, the lungs, the liver, the spleen, the bones, and the kidneys, but from a study of the spontaneous tumor alone, it would be impossible to say what the conditions were that determined this particular distribution of metastases. The picture presented might be regarded as a matter of chance or as an indication of a predilection of the cells of this tumor for growth in certain organs or as a condition determined by the mode of cell distribution. On the other hand, it might be viewed as an indication of resistance of different organs or tissues to the growth of the tumor cells or as a combination of all of these factors and perhaps of others. At any rate, these are among the possibilities that have to be considered, and the results obtained from the study of transplanted tumors may be analyzed with a view to determining how far the influence of such factors can be traced or the extent to which they have dominated the behavior of the tumor.

The incidence and distribution of metastases, the characteristics of the growth in different locations, and the circumstances of their occurrence, as well as the fate of lesions in different organs were described in the preceding papers of this series (5, 6) and will be used here as the basis for a discussion of factors that have influenced the occurrence and distribution of metastases.

DISCUSSION.

As regards the rôle of the tumor cell, it may be said that the picture presented by the original animal has never been duplicated. During the first twenty generations, no distant metastases were obtained in normal animals inoculated either in the skin or the subcutaneous tissues, and, although a great variety of lesions was produced by intratesticular inoculation, in no instance was there such a preponderance of metastases in the liver, spleen, and bones. From this, it is at once apparent that the tumor *per se* was not the sole cause for the picture presented by this animal but that there were other factors peculiar to the animal itself that played a part in determining the result.

In like manner, it must be apparent that, in as far as any inherent quality of the tumor cell itself is concerned, all animals and all organs or tissues may be regarded as being upon an equal footing Still, it was found that even animals of the same series might show the greatest diversity in results so that, while the tumor cell must be considered as an essential element in the equation, it is obvious that its activities were limited by outside influences which were capable of producing an almost endless series of variations even though all experimental conditions were virtually constant, except those that concerned the individuality of the host

The problem of metastasis may then be narrowed down to two main groups of factors, namely, accessibility of the part to tumor cells in a viable condition and the ability of the cells to grow in a particular organ or tissue or, so to speak, phenomena of cell transport and of cell nutrition in its broadest sense. The key to the solution of the problem lies, therefore, in conditions that determine distribution of cells, conditions that affect the viability of the cells during transport, and the ability of the cell to grow where it becomes lodged.

As has been pointed out, the mode of growth of this tumor is such as to render it practically certain that some dissemination of cells occurs early and in all animals with actively growing tumors and that cells are distributed to distant parts of the body by two separate and distinct paths, the blood stream and the lymphatics. However, mere entrance of tumor cells into the blood stream or lymph channels is not sufficient to insure the development of metastases. In fact, growth was rarely obtained by intravenous inoculation of normal animals, and there was virtually a complete failure of the cells to grow in distant organs of upwards of 40 per cent of the animals inoculated in the testicle, although it is known that, in all such animals, there were tissues in which the cells might have grown with ease provided they could have reached such places in a viable condition Under ordinary circumstances, therefore, it is obvious that many cells entering the blood stream or the lymphatics are disposed of in such a way as to prevent their growth, and apparently this mechanism is more efficient in some animals than in others.

On the other hand, it has been seen that, when metastases do occur, a certain degree of order and of system prevails as regards the time and circumstances of their occurrence, the distribution of the lesions, the character of the growth in a given location, and the reaction displayed by the surrounding tissues. Such a course of events is not altogether a matter of chance but is an expression of the action of conditions which have some part in shaping the course of the disease.

The nature of the factors concerned in these processes may be best appreciated by considering first those animals in which metastases occur as distinguished from those in which no metastases develop. If we may again make use of the course of disease in different groups of animals as an index of general resistance, it would be safe to assume that those animals in which the disease pursues a fulminating course are the ones that are most indifferent to the tumor and these were the animals that showed the most widespread distribution of metastases. They accounted for the wave of maximum incidence and of maximum distribution that occurred at about the 6th to the 7th weeks after inoculation.[1]

If one considers the incidence and distribution of metastases in all classes of animals during the first 6 to 8 weeks after inoculation, it will be noted that the peak of metastatic involvement was reached in practically all those organs and tissues which are not directly in the path of the lymphatics leading upward from the primary tumor. During the first 4 or 5 weeks, the lungs showed the highest incidence of metastases, followed by the suprarenals, the kidneys, the liver, and the heart, in the order given, with such organs as the eyes, the muscles, the superficial lymph nodes, the bones, the hypophysis, and the skin forming a second group in which the order of frequency was less definite.

The sequence in development of metastases in this group of organs suggests a distribution of cells by the blood and the order of incidence conforms fairly closely to that usually given for the lodgment of blood emboli, with a notable absence of metastases in such organs as the spleen and the central nervous system. (The area supplied by the mesenteric arteries is here omitted on account of the possibility of confusing blood- or lymph-borne metastases with implantations.) There is not an absolute agreement, however, but the evidence is sufficient to show that under favorable conditions, cells entering the blood stream may pass the pulmonary capillaries and be distributed to various parts of the body in a viable condition and that, in such cases, the location of metastases may be determined largely in accordance with principles of embolic distribution.

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[1] Part I, Text-fig. 2.

It is obvious, however, that mere mechanical distribution cannot determine growth. From what has been learned of the ability of the tumor to grow in different tissues by the use of direct inoculation and by the study of metastases, it is obvious that the possibilities of growth in any location are contingent upon the availability of nutritive substances. If the factor of animal resistance could be eliminated, the tumor would grow best in those organs where food is most abundant, but under ordinary circumstances, growth is modified to a greater or less extent by the local reaction, and this varies in different organs or tissues so that the picture actually presented in any given instance represents a resultant of the two factors. Thus, in the most fulminating cases of malignancy, cells may be distributed in large numbers to nearly every part of the body and they may grow almost anywhere, but they appear to grow best where food is most abundant. For example, in cases of this type, one almost invariably finds the most extensive growth in the liver. The lesions in other organs may be almost as numerous, but even in such organs as the kidneys they do not show the same activity of growth. In fact, they may be so small as to be barely perceptible. In the same way, metastases in the lungs, the skin, and subcutaneous tissues rarely showed a rate of growth equal to that of metastases in parenchymatous organs, lymph nodes, or muscle even though there was comparatively little local reaction to interfere with their nutrition.

In like manner, while cells are distributed both by the lymphatics and the blood, lymphatic distribution is a relatively slow process and any effect that it might have in determining the location of metastases may be forestalled to a great extent by a more rapid distribution of cells through the blood.

In certain instances, therefore, the development of metastases may become a function of time and of nourishment. This is especially true in animals with the most malignant tumors. Such animals are essentially indifferent; viable cells are distributed mechanically to all parts of the body, but on account of differences in the food supplied in different organs, certain lesions grow rapidly and death occurs before the cells in other locations have had an opportunity to develop.

In animals that show an increase in resistance with the progress of the disease, the distribution of cells and the growth of metastases are determined more and more by positive factors of animal resistance. As long as resistance is comparatively low, cells may be widely distributed and growth may take place in many parts of the body, while the rate of growth is still determined largely by the nourishment provided in a given tissue. But in more resistant animals, or as resistance increases, the distribution of cells is more restricted and the actual food supply of an organ becomes of less moment. Under these circumstances, the important factors in the development of metastases are accessibility and availability which are determined on the one hand by the resistance encountered in the medium of cell transport, plus mechanical factors of distribution, and, on the other, by the local resistance to growth in different organs or tissues.

The effect of these conditions is to alter the general scheme of liability to metastatic involvement. Cell distribution tends to be limited more and more to those organs with the most abundant and direct blood supply or to parts that are accessible to the spread of the growth by the lymphatics, while a further restriction is imposed upon the development of metastases by the ability of the cell to grow in such places as are still accessible.

The first effect of these conditions is to produce a more slowly progressive disease with greater opportunity for the development of metastases, so that rapidly progressive cases of malignancy in animals that survive 6 or 8 weeks may show a more extensive distribution of metastases (gross) than those that die within the first 4 or 5 weeks, while the relative extent of the growth in different organs also undergoes some change. Thus, the growth in the kidneys was usually more abundant than that in the liver, and comparatively large lesions were found in a number of locations, while in cases of more acute death, they were either absent or extremely small. Hence in animals of this class, a more uniform distribution of lesions and a more uniform growth were the outstanding features of the picture presented as compared with that seen in the most fulminating cases of malignancy.

As the disease became more chronic, however, the order of metastatic involvement underwent a very decided alteration. The relative incidence of metastases in such organs as the lungs, the kidneys, the suprarenals, the liver, the heart, the eyes, the muscles, the superficial lymph nodes, the bones, the hypophysis, and the skin and subcutaneous tissues suffered a gradual or an abrupt reduction, the character and extent of the change varying with different organs. At the same time, there was an increase in the relative incidence of metastases in the deep lymphatics, in the retroperitoneal tissues, and in the mediastinum, and the change in one direction virtually compensated for that in the other. Finally, a terminal increase in the incidence and distribution of metastases occurred, so that in animals that succumbed after a prolonged course of disease, one might again find the same widespread distribution of lesions as in fulminating cases of malignancy, and this feature of the disease is deserving of especial emphasis.

Much of the change in the picture presented by these animals might be accounted for upon the basis of the principles of embolic distribution and what is known as to the ability of the cells to grow in different tissues. Such organs as the lungs, the kidneys, the suprarenals, and the liver still showed a fairly high incidence of metastases, and the decrease that occurred here or in other organs might be attributed to local tissue resistance. While this explanation might serve in the case of such organs as the skin and subcutaneous tissues, the lungs, the kidneys, and the liver, it would not hold in the case of the suprarenals, the hypophysis, the eyes, the bones, the superficial lymph nodes, and the muscles any more than it would in the case of the deep lymph nodes, the retroperitoneal tissues and the mediastinum where the incidence of metastases was upward rather than downward. In fact, there was no evidence of any local resistance to the growth of the tumor in either the suprarenals or the hypophysis, and the resistance encountered in the ciliary body, the lymph nodes, and the bones was comparatively feeble.

In view of the number of actively growing tumors in these animals, there can be no doubt that cells entered the blood stream and the lymphatics as in any other animals with actively growing tumors, but, for some reason, few of the cells entering the blood arrived at their destination in a viable condition or else they were removed from the circulating blood by some mechanism which interfered with their normal distribution. Under these circumstances, blood-borne metastases were, as has been said, confined to those organs with the most abundant and direct blood supply, while lymph-borne metastases either retained a comparatively high level of incidence or showed an actual increase. Eventually a point was reached at which the growth appeared to spread almost entirely by the lymphatics, and few or no metastases were found, except in the retroperitoneal tissues and the mediastinum.

While in such animals as these the resistance to growth on the part of the tissues is doubtless greater than that of animals in which the disease pursues an acutely fatal course, it is obvious that conditions affecting cell transport play an even greater part in determining distribution and growth of metastases, for, as has been pointed out, there are still a number of organs in which the tumor might grow with ease provided the cells could reach such locations in a viable condition. This applies especially to the suprarenals and the hypophysis, where, as far as is known, there is virtually no local resistance and tumors never heal, except as a result of vascular occlusion. Moreover, as we have been able to show by direct inoculation, the suprarenals may still retain a degree of susceptibility even in a recovered animal.[3]

It is clear, therefore, that, in animals with a high native resistance or whose resistance increases at a rapid rate in consequence of a tumor inoculation, the incidence and distribution of metastases are primarily functions of cell distribution. In this instance, however, it is evident that we are no longer dealing with purely mechanical principles of cell embolism but with some positive factor of resistance analogous to that concerned in preventing the growth of cells injected intravenously into normal animals. That such is the case is shown by the fact that animals in which the disease has pursued a chronic course with metastases confined largely to lymphatic paths, may in the end develop numerous metastases in other parts of the body with showers of microscopic lesions in the vessels of such organs as the lungs, the kidneys, and the liver, indicating a gradual or sudden collapse of some mechanism which hitherto had prevented any extensive dissemination of viable cells by the blood. It will be recalled that this was the condition found in the animal with the spontaneous tumor.

If one pursues the line of reasoning that has been followed thus far it will be found that many of the phenomena of metastasis may be explained upon the basis of the general principles of cell distribution and of growth which have been outlined above. As long as we are dealing with cases of high malignancy, the situation is comparatively simple, but as we pass from animals of this class to those that display a higher degree of resistance, we are confronted by increasing difficulties, and in any event, there are peculiarities of incidence and of distribution or of absence of metastases from certain organs for which it is difficult to find a satisfactory explanation. This applies to such conditions as the centralization of metastases, the low incidence of metastases in the uninoculated testicle, the spleen, and the thyroid and the complete absence of metastases from the central nervous system.[3] In all such instances, however, there is some evidence as to the nature of the cause that is responsible for the condition presented.

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[3] Pearce, L., and Van Allen, C. M., unpublished experiments.

Attention has been called to the fact that metastases in general diminish in frequency with the distance from the body axis, or, where the blood is the medium of cell transport, other conditions being equal, they diminish with the distance from the heart. This indicates one of two things, either that the tumor cells tend to become lodged before they are carried very far or that the further they are transported, the less liable they are to reach their destination in a viable condition In either case, it is obvious that they are subjected to some influence in the blood which tends to interfere with their growth, and the effects of this influence are more apparent under some conditions than under others. It is certain, however, that this is the main factor concerned in the centralization of metastases.

A similar condition appears to be responsible for the low incidence of metastases in the uninoculated testicle. The occurrence of so few metastatic lesions is difficult to reconcile with our knowledge of the growth of cells that are mechanically introduced, and it may be that the tortuous nature of the vessels and their mode of entrance into the testicle give an added protection against the entrance of tumor cells This assumption is supported by the fact that metastases do occur with greater frequency in the cord and epididymis and that most of the cases of testicular metastases recorded were in animals with very malignant tumors.

[3] Since this statement was written, we have encountered one case of cerebral metastasis in which the growth apparently arose from the choroid plexus in the right lateral ventricle.

The problem presented by the thyroid is totally different. Persistent growth of tumor cells and the formation of visible metastases in the thyroid occurred only in animals whose resistance was of the lowest order. This cannot be accounted for on the basis of accessibility, since, with its abundant and fairly direct blood supply, it is reasonably certain that viable cells are constantly being brought to the thyroid in large numbers. Some of them make abortive attempts to grow but are almost immediately submerged beneath a massive chronic inflammatory reaction such as is not seen in any other organ or tissue. In fact, this reaction is maintained in some animals after it has practically failed elsewhere, and apparently it is the tangible means of suppressing tumor growth in this organ.

Neither of the above explanations is sufficient to account for the low incidence of metastases in the spleen. Tumor cells undoubtedly reach the spleen in large numbers, but for some reason they do not grow. There is no marked granulomatous reaction such as is believed to be a controlling factor in some organs, and it is equally difficult to attribute the immunity of the spleen to the presence of large amounts of lymphoid tissue, since the tumor metastasizes by way of lymphatics as well as the blood stream, or even when it cannot metastasize by the blood, and lymph nodes themselves are peculiarly favorable sites for the growth of the tumor. This, of course, is the paradox which renders it difficult to assign a rôle of especial importance to lymphoid tissues *per se* in combating such diseases as tuberculosis, syphilis, and cancer. In all these conditions, the causative agent of the disease lodges and grows in lymphoid tissues by preference, as it were, or as though it enjoyed a protection which was not afforded elsewhere, and, as is known, the causative agent of some of these diseases does survive longer in lymph nodes, spleen, and bone marrow than in other tissues. It is obvious, therefore, that the defensive function of these tissues can be exercised only in conjunction with other elements or tissues. In other words, there are factors beyond the lymphoid tissues themselves.

In a few instances in which microscopic lesions were present in the spleen, the growth was located at the center of the Malpighian bodies. This may be no more than an indication of the point at which aggregates of cells are most apt to become lodged, or it may mean that the conditions for growth are better here than in other parts of the spleen. While we do not pretend to know the exact causes for the failure of the cells to grow in the spleen, the evidence available would seem to indicate that anatomical or circulatory conditions are chiefly responsible in that the presence of a system of venous sinuses may afford an exceptional opportunity for the disposal of tumor cells.

The complete absence of metastases from the tissues of the central nervous system is still more perplexing. When inoculations are made directly into the substance of the brain, the tumor grows very actively and there can be no doubt that, with actively growing tumors in other parts of the body, large numbers of cells find their way into the capillaries of the central nervous system. Still no growth takes place Hence it would seem that an especially efficient mechanism must bar their entrance to these tissues. This is not of the nature of a cellular reaction but apparently conditions exist which inhibit the growth of cells within the capillaries to a greater extent than in any other tissue of the body. The evidence available indicates that the mechanism concerned is the same as that which prevents the growth of cells that gain access to the blood stream of highly resistant animals but that its action is greatly intensified in the central nervous system

It will be seen, therefore, that from whatever angle the problem of metastasis is approached, the crucial factor in determining widespread or restricted distribution of metastases or the presence or absence of metastases in any particular location is the factor of cell transport. The ability of the cells to grow is sometimes the decisive factor, but more often this is of secondary importance. The first line of defense is clearly the medium of cell transport, and in seeking an explanation for any given condition we are almost invariably forced to recognize the existence of some controlling mechanism in the blood or other body fluids whose action permits or prevents the occurrence of metastases on the one hand and regulates their distribution on the other.

The tumor may metastasize by the lymphatics when cells are incapable of surviving in the blood but, as long as the cells are distributed entirely by the lymphatics, the disease progresses extremely slowly and is stereotyped in form; variation in the picture presented, widespread metastases, and high malignancy, are largely functions of blood transport.

Thus far, no effort has been made to indicate the nature of the factor in the blood that regulates cell transport. We have dealt with animals in which this factor was either inoperative or only incompletely developed but we come now to a consideration of those animals that show a complete absence of metastases. Once it has been shown that the cells of a malignant tumor are capable of surviving the process of transplantation and of growing in almost any part of the body, it is not so difficult to understand how cells from an actively growing tumor of a malignant character might gain lodgment in distant parts of the body and establish secondary foci of tumor growth as it is to understand why no secondary growths develop in other animals with tumors of the same character and equally active as to growth and local attributes of malignancy. This aspect of the subject is obviously of more far reaching importance than the presence of metastases and an understanding of one condition is essential to an understanding of the other.

It may be said at once that the reasons for the complete absence of metastases are not definitely known, but since in all animals there are numerous locations in which the tumor will grow with perfect ease, the failure of metastases cannot be accounted for on the basis of tissue susceptibility or local tissue resistance. There are, however, many facts which indicate that such a condition might be brought about by a reaction of the blood and of other body fluids. In approaching the problem from this point of view, one has first to consider the attitude of the animal toward the tumor cell as indicated by reactions which follow its introduction into the animal body. When cells are introduced into the tissues, they excite a reaction which is comparable in all respects to a foreign body reaction. There is an abundant growth of granulation tissue and an effort at encapsulation which may or may not succeed in checking the growth of the tumor, and subsequent phases of the reaction vary accordingly.

If the cells are injected into the peritoneal cavity, much the same thing happens. As has been stated, tumors rarely develop; the cells gradually become collected into small masses surrounded by fibrin and may remain free in the cavity for some time or become attached to the peritoneal surface and walled in by the growth of granulation tissue. In other words, the cells are disposed of as though they were some organized foreign material of a relatively indifferent character.

The fate of cells injected into the blood stream is not definitely known but the indications are that they are disposed of in much the same way as cells that are introduced into the peritoneal cavity. At any rate, they rarely give rise to tumors, and since they are elements that are foreign to the blood, it is not unlikely that they tend to induce a formation of fibrin or of thrombi, such as Schmidt (7) has described in cases of human cancer, and are in this way prevented from obtaining the nourishment necessary for their growth.

If, therefore, we may be guided by the attitude of the animal toward the tumor cells, it is obvious that although they are products of rabbit tissues, the rabbit treats them as though they were foreign elements either because they have suffered some alteration which has affected their relations with normal tissues or because they are misplaced and brought into relations where they have no normal place. So long as we recognize the fact that the tumor cell arouses an antagonistic reaction, it is immaterial which of these explanations is accepted.

Again, if one analyzes this reaction in detail, it will be found that it presents many of the characteristics of an antigen-antibody reaction. This is seen in the increased resistance which occurs with the growth of the tumor, in the periodicity of growth and of resolution, and in the occurrence of the phenomenon which has been described as a crisis and reminds one strongly of a local anaphylactic effect.

In this connection, attention may also be called to the alteration which takes place in the coagulability of the blood and is intimately associated with clinical malignancy on the one hand and the incidence and distribution of metastases on the other. In fact, one of the most striking features of highly malignant tumors is the marked decrease in the coagulability of the blood which, in cases of fulminating malignancy, may extend even to incoagulability, while there is a change in the reverse direction in animals whose resistance is increased (3).

There is no evidence, however, that the reaction of the tumor animal is entirely specific. On the contrary, it is known that it is in part non-specific, but it is also known that resistance to the tumor may be greatly increased by non-specific stimulation.

The significance of these facts may be better appreciated if one applies them to the problem of metastasis, bearing in mind that conditions which favor or prevent the occurrence of metastases cannot be considered entirely apart from the primary tumor itself and from processes which are brought into activity by its presence. As has been said, cells that are mechanically introduced into the circulating blood of normal animals are usually disposed of in such a way as to prevent their continued growth, and there is evidence that a similar reaction takes place when cells are discharged into the blood stream from a growing tumor. But, in view of the fact that the reaction (resistance) of normal rabbits differs with all forms of inoculation, it would not be expected that all animals would display the same sensitiveness to the presence of these cells or the same capacity for their disposal. At the beginning, therefore, other conditions being equal, the fate of the cells discharged into the blood would depend primarily upon the existing state of these two factors. With the continuation of the process, the cells and products of tumor growth acting as antigen, one of several conditions might be induced which may be illustrated by concrete examples of what actually does occur in different forms of the disease.

In the first place, many animals possess a high threshold resistance to the tumor cells in both the tissues and the body fluids. If, in addition to this, a gradual or rapid increase in the capacity for cell disposal occurred as a result of a process of automatic immunization, paralleling the reaction to the primary growth, then regression and absorption of the primary tumor would occur without the production of metastases.

In other animals, either with or without an increase in the native capacity for the disposal of cells, a sensitization might take place which would express itself in either or both of two ways: first, by a prompt disposal of cells discharged into the circulation, and second, by a kind of local anaphylaxis due to the action of antigen and antibody, if we may term them such, in the zone of concentrated antigen within and immediately surrounding the primary tumor. A condition of this kind does occur in many animals, giving rise to suppression of the primary growth by what has been termed a crisis. This condition, it will be recalled, is characterized by the sudden development of edema, which usually persists for a short time and then rapidly disappears. The essential element in this reaction appears to be the formation of coagula of some kind in the smaller vessels within or immediately surrounding the growth, and possibly in the drainage lymphatics, which obstruct the flow of blood and lymph to a sufficient extent to cause an edema, and, while the obstruction is only temporary, it is usually sufficiently enduring to impair seriously the vitality of the growth and not infrequently produces a complete necrosis of the tumor. This reaction is identical in its obvious characters with that which occurs in syphilitic infections.

It is important to bear in mind that this type of reaction occurs with the most actively growing tumors, that while the time of occurrence varies with the rate of growth in different series of animals, it is remarkably frequent at or near the end of the 3rd week after inoculation. With a growth of this type, one would expect a maximum discharge of cells into the blood and lymphatics. Still, recovery frequently takes place without the development of metastases.

The exceptions to this rule are equally interesting. In some animals, the crisis is, as we have termed it, ineffectual, in that after partial regression, there is renewal of growth in the primary tumor, or, in spite of a critical reaction of considerable degree, there is little or no regression but the tumor continues to grow. In many instances, the growth of the primary tumor after crisis is comparatively slight, but the occurrence of an ineffectual crisis almost invariably signifies high or even fulminating malignancy which is the exact opposite of the condition which exists in the case of an effectual crisis. In such instances, it would seem that the animal expends a great part of its resources for preventing the successful implantation of cells. At all events, metastases appear in all parts of the body and grow with comparatively little opposition, indicating either that these animals possessed a feeble resistance from the beginning or that their mechanism of resistance to both cell transport and growth were affected at the same time and in the same manner.

This brings us to a third possibility as regards the behavior of animals towards the presence of tumor cells; namely, relative indifference with sluggish response. For a time, such animals would permit the growth of the tumor and the development of metastases in favorable locations, but as they became aroused, resistance would be increased and both metastases and primary tumor suppressed. In some instances, however, metastatic growths might remain in such places as the suprarenals or the eyes, and we would have the paradox of an immune (?) animal with a living tumor which could not be suppressed but with other tissues protected either by the resistance in the medium of cell transport, or by a local tissue resistance.

The same conditions might be brought about by the existence of a relatively low threshold resistance with a reserve capacity sufficient to compensate for this defect. It is difficult to say whether the start that the tumor gets in such cases is due to indifference or to a low threshold resistance. In some instances, it appears as though the resistance of the animal were taxed to its uttermost, while in others, the tumor grows actively for a time but is checked abruptly and with no apparent effort on the part of the animal, which suggests that resistance was merely dormant.

A further possibility is that presented by the animal in which there is a persistence of an inactive or sluggish primary tumor with metastases distributed along the lymphatic paths of the body axis and after long periods of time gradually involving other organs of the body. The picture presented in these cases is clearly indicative of an initial *metastasis by way of the lymphatics*. This would imply an immunity to blood transport, since, when this path is open, metastasis is certain to occur by this route.

As has been pointed out, animals of this class not infrequently show a terminal systemic involvement which clearly indicates a collapse or exhaustion of the entire mechanism of resistance.

Finally, there is a group of animals that is either almost insensible to the presence of tumor cells or incapable of dealing with them either in the tissues or body fluids and the tumor progresses to a fatal termination with scarcely any opposition.

While, therefore, the ultimate cause may not be known for the absence of metastasis in animals in which it is reasonably certain that numbers of cells enter the blood and the lymphatics, it is highly probable that the conditions which have been outlined play a very considerable part in determining both the presence and the absence of metastases. In fact, it appears that the introduction of tumor cells into a normal animal acts in many ways as a parenteral injection of a foreign protein or of organized foreign material. But, in addition, the tumor cell is a living and aggressive agent which tends to perpetuate itself and in so doing arouses an antagonism on the part of the host which, within the capacity of the animal, increases with the growth of the tumor or until one or the other prevails. This reaction is in many respects analogous to that seen in certain protozoan infections, which is not surprising since in both cases the causative agent is a highly organized animal cell.

If we adopt this attitude toward the tumor, it will be seen that the outcome of the interaction between tumor and host is determined by a chain of processes which are set in operation at the time of inoculation. Some normal animals possess the necessary capacity for suppressing the primary growth and at the same time are capable of disposing of any cells that may gain entrance to the blood stream or lymphatics. But, in most instances, the threshold resistance is comparatively low and increased powers of resistance are called into being by a process akin to immunization.

This reaction occurs in close association with the growth of the tumor but is directed against the tumor cells wherever they may be found. In like manner, the expression of resistance varies according to the *means available for preventing the growth of cells in different* locations. In all cases, however, there is an encapsulation in some form which deprives the cells of the nourishment necessary for their growth and at the same time hinders their distribution. Where viable cells gain lodgment in the tissues, a granulomatous reaction occurs and is aided in its action by the formation of coagula in the vessels that supply nourishment to these tumors. In the blood, tumor cells appear to be disposed of in the same way as any organized foreign material that cannot be phagocyted. They are surrounded by fibrin and filtered out of the circulating blood or are walled in by the formation of a thrombus.

Essentially the same thing occurs in the serous cavities and in the lymphatics, but the conditions which favor this method of disposal are not the same in all parts of the body. In some places, anatomical conditions are doubtless of importance, as in the case of the spleen; in others, the nature of the tissue aids in determining the end-result, as in the lungs and the skin on the one hand, where growth is difficult even under the most favorable circumstances, and in the liver and kidneys and the suprarenals, the hypophysis, the lymph nodes, the bone marrow, and the eyes, on the other, where food is either abundant or the local resistance to tumor growth is comparatively feeble.

Finally, it is clear that in certain organs, such as the central nervous system, there is an additional factor which aids in the disposal of cells in the circulating blood. It is known that the tissue of the central nervous system contains substances (cephalin) which are among the most powerful coagulants, and it appears not unlikely that the freedom of certain organs from metastases may be attributable to the action of some substance which facilitates coagulation about the cells contained in the blood passing through these organs.

There may be other processes concerned in the disposal of tumor cells, but there is no evidence to indicate that such is the case and the known facts do not require such an assumption.

Little is known of processes of protection or immunity involving phenomena of coagulation such as appear to be operative with this tumor. The phenomena described are analogous to what has been observed in so called agglomeration reactions of protozoa (trypanosomes and blood spirochetes), and it is not improbable that similar processes may be concerned in freeing the blood of foreign cells or organisms of many kinds

Pathologists have realized that some humoral factor must play a part in the resistance to malignant tumors and to protozoan diseases, but attempts to demonstrate protective principles have been confined so largely to studies of serum, as distinguished from plasma or whole blood, that the importance of phenomena of coagulation has not been fully appreciated.

It is not known how the changes in coagulation are effected, but there are a number of possibilities to be considered. Among these are the loss of the calcium that occurs in malignant disease and variations in fibrinogen and thrombokinase, such as occur in practically all forms of parenteral injection of foreign proteins, both of which have a direct bearing upon the problem of tumor resistance. However, it will require further investigation to determine the nature of the change that at one time enables the blood to render inactive all the cells that enter and at another leads it to behave as an inert medium distributing the cells to different parts of the body in a purely mechanical way.

CONCLUSIONS.

With a knowledge of what takes place at the site of inoculation, in the medium of cell transport, and at the point of lodgment of cells in different parts of the body, the problem of metastasis is greatly clarified. It is possible to explain both the presence and the absence of metastases as well as peculiarities of incidence and of distribution upon the basis of established facts which in no wise conflict with what has been determined in the case of other tumors. On the contrary, if this conception be adopted, it will aid materially in clarifying our understanding of phenomena of metastasis or of tumor growth in general.

Once it is granted that the tumor cell is capable of exciting an antagonistic reaction on the part of the host, either in virtue of a change in the cell itself or of its entrance into an environment where it has no normal place, the problem immediately resolves itself into a consideration of the consequences entailed in accordance with the general scheme that has been outlined above.

When viewed from this standpoint, the current conception of a malignant tumor as a lawless growth (autonomous) is hardly tenable. It may be viewed as an abnormal growth resulting from a disturbance of a growth-regulating mechanism and is lawless in the sense that it tends to override existing processes of restraint, but its behavior is that of a living and aggressive foreign agent, and all the phenomena associated with its presence are susceptible of analysis upon the basis of the same principles of pathological action that are applicable to such conditions. Even the distinctions that are recognized in the mode of metastasis of different classes of tumors are explainable upon this basis, not as expression of any inherent tendency on the part of the tumor to spread by any particular path but merely as results of following the line of least resistance. Naturally this would vary according to the origin of the cell. Thus cells of a sarcoma are not so far removed genetically from blood elements as are epithelial cells and hence might arouse less antagonism if they entered the blood stream and, under ordinary circumstances, would be more likely to be distributed by this path than the cells of a carcinoma. On the other hand, metastasis by way of the lymphatics is probably not due to any selective activity but is a matter of necessity in many instances, or until the blood resistance has been reduced to such an extent that cells entering the blood stream are no longer destroyed.

This discussion of the phenomena of metastasis leaves many questions still untouched. The foremost of these are the causes for the wide variations in the incidence of metastases (28 to 100 per cent) in different series of animals and for such differences in the behavior of individual animals as have been described. These subjects have a more intimate connection with conditions that affect malignancy or animal resistance and will be taken up in the next papers of this series which deal with variations in malignancy and factors that influence animal resistance.

SUMMARY.

Data bearing on the occurrence and distribution of metastases, as recorded in the preceding papers of the series, are analyzed with reference to the parts played by the tumor cell and by conditions which influence cell distribution and cell growth.

It is shown that, while the tumor cell is an essential element in the production of metastases, its influence is relatively constant, and that the prime factors responsible for variations in the occurrence and distribution of metastatic growths are conditions that influence cell distribution or that affect the viability of the cell during transport, and conditions that affect the nutrition of the cell wherever it may become lodged.

It is further shown that both the presence and the absence of metastases, as well as peculiarities of distribution and of growth, are susceptible of a comparatively simple explanation upon the basis of well recognized principles of pathological action. In brief, it was found that, while the tumor cells might be distributed by either the blood stream or the lymphatics, the mode of distribution and the fate of the cells were determined in accordance with the same general principles that are applicable to the distribution and disposal of foreign cells introduced into the animal body. It was pointed out, however, that in applying these principles to tumor metastasis, it was necessary to recognize the fact that tumor cells bear a definite relation to the tissues of the host and that they are living and agressive agents. Hence, the

reaction that they arouse differs in some respects from that produced by inert or unorganized foreign material or by living cells of a foreign species.

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STUDIES ON THE NASOPHARYNGEAL SECRETIONS FROM PATIENTS WITH COMMON COLDS.

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The experimental studies on the incitant of common colds in man to be reported in this paper were begun shortly after the investigations on influenza by Olitsky and Gates [1-3] were undertaken. They were initiated by the desire to check or control the latter studies and to determine, if possible, any causative agent in the nasopharyngeal secretions derived from persons suffering from common colds. This work has now extended over 4 years, and, while still incomplete, our purpose is to state only the results which we regard as established experimentally.

Source of Materials.

It is obvious that patients subjected to study should be selected carefully, for the pathognomonic symptom, rhinitis, can be induced by exposure to cold and by other physical and chemical agents, and is a sign of several affections, such as hay-fever, influenza, measles, and other exanthemata. Only such affected individuals were chosen who could trace their illness to exposure to a previous case of common cold within 3 or 4 days; in other words, only those who had an infectious condition and showed at the same time the typical syndrome of the disease. This consists of premonitory malaise and dryness of the nasal passages, followed by sneezing and discharge of a profuse, thin, irritating secretion from the nose. Conjunctivitis of a mild degree with

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[1] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 125, 361, 373, 713, xxxiv, 1; 1922, xxxv, 1, 553, 813.

[2] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxvi, 501.

[3] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxvi, 685; 1923, xxxvii, 303; *Science*, 1923, lvii, 159.

lacrimation is usually present and in some persons slight headache. The temperature is either not elevated or only slightly so and in general the constitutional reaction is negligible. On the 2nd or 3rd day the nasal discharge diminishes in quantity, becomes first mucoid and then purulent; the mild constitutional symptoms, if present at the beginning, disappear, and in uncomplicated cases, complete recovery ensues after 4 or 5 days. In the purulent stage, however, secondary localizations may occur by extension of the inflammation to the ear, inducing otitis media, or to the paranasal sinuses, or to the larynx. In these instances, the microorganisms usually found associated are the staphylococcus, streptococcus, pneumococcus, and Friedländer bacillus groups, the meningococcus, *Micrococcus catarrhalis*, and Pfeiffer's bacillus.[4,5]

Ringer's or 0.85 per cent saline solutions were employed to wash the nasopharyngeal secretions from cases of common colds. These washings were collected from 40 patients in the early hours (from 3 to 20) of the uncomplicated illness In addition, washings were obtained from 30 individuals, supposedly free from common colds, and were employed as controls. In general the methods used in the following experiments were those described in the series of papers on influenza by Olitsky and Gates[1-3]

Transmission Experiments

Common cold is a mild disease in man, constitutional reaction is either lacking or is transitory and inconsequential. There is no definite, measurable criterion of a positive transmission to rabbits the animals employed in the experiments. The symptoms of nasal discharge and sneezing cannot be regarded as critical in rabbits, for these animals, as a future report will show, develop snuffles spontaneously in association at times with an underlying chronic paranasal sinusitis. Thus, after nasal swabbing of thirteen rabbits with filtered and unfiltered nasopharyngeal washings from fresh cases of common cold in man, six showed, within 2 to 4 days thereafter, symptoms of nasal

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[4] Lacy, G R., *J. Lab and Clin. Med.*, 1918, iv, 55

[5] Crowe, S. J., and Thacker-Neville, W S., *Bull Johns. Hopkins Hosp.* 1919, xxx, 322.

discharge, a proportion which prevailed, however, among an equal number of animals swabbed with control materials. Similarly, the intratracheal injection into fifteen rabbits of the unfiltered nasopharyngeal washings derived from ten cases of early common colds was followed by no constant or characteristic effects. Although the lung tissue of injected rabbits was reinoculated into two successive series of animals, the results of these intratracheal inoculations showed that some animals gave a polynucleosis with frank lobar pneumonia, from the lungs of which either the pneumococcus or streptococcus or rabbit septicemia bacillus could be recovered in pure culture, others a mononucleosis without lung involvement, and the remainder, inconstant effects. Similar results were obtained from the washings of six supposedly normal individuals injected intratracheally into eleven rabbits.

The conclusions to be drawn from our attempts at transmission to rabbits are that, in the absence of a suitable criterion for a positive transmission or because of the mildness of the disease, these animals failed to show any definite effects. On the other hand, it is apparent that experimentally the nasopharyngeal secretions of early cases of common colds are distinct from those of influenza, since the latter, as already demonstrated,[1-3] induce constant and striking effects both in the blood and on the lungs of rabbits.

Transmission to Man.

It is apparent that a disease of so mild and transitory a nature as the common cold could best be studied, in as far as transmission of any possible incitant is concerned, in man. Before describing the experiments the previous work of Kruse,[6] in 1914, and Foster,[7] in 1915-16, should be mentioned.

Kruse diluted the nasal secretion of an assistant ill with a common cold with 15 times its volume of saline solution and then filtered the material through a Berkefeld candle. A few drops of the filtrate were instilled into the nose of each of twelve men. In 1 to 3 days, four of them showed common colds. On repetition, the nasal discharge from a case of common cold was taken up in 20 volumes of saline solution and instilled into the nose of each of 36 volunteers. In 1 to 4 days fifteen, or 42 per cent, became ill with this disease. Kruse concluded that the incitant of common colds is a 'filterable microorganism, although he was unable to demonstrate either morphologically or by culture a formed, living and multiplying element.

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[6] Kruse, W., *Münch. med. Woch.*, 1914, lxi, 1547

[7] Foster, G. B., Jr., *J. Infect. Dis.*, 1917, xxi, 451; *J. Am. Med. Assn.*, 1916, lxvi, 1180.

Foster later believed that he confirmed the findings of Kruse The nasal secretions from cases of common colds within 24 hours after the onset of the first symptoms were suspended in 10 cc. of saline solution and, after shaking, the homogeneous suspension was filtered through a Berkefeld N candle From 3 to 6 drops of the filtrate were instilled into both sides of the nose in each of ten men. "7 developed clear cut and definite symptoms of acute coryza; 2 reacted questionably, while 1 remaining case exhibited no symptoms. There was an incubation period of 6 or 8–30 hours. . . . The duration of the symptoms varied 3–6 days—usually 5." In addition, Foster stated that he has cultivated in fresh tissue-ascitic fluid medium from eleven cases of the natural disease and from five of experimental common colds a minute anaerobic microorganism similar in many respects to the "globoid bodies" of Flexner and Noguchi[8]

The present experiments were made on nineteen volunteers with the washings collected from eight patients. The mode of procedure differed somewhat from that of Kruse and Foster. Instead of the nasal discharges, we obtained the nasopharyngeal secretions by washing with 25 to 30 cc. of either Ringer's or 0.85 per cent saline solutions.[1-3] The suspension was shaken and then filtered through a Berkefeld V or N candle The clear filtrate free from ordinary bacteria, as determined by cultivation tests, was applied to cotton applicators 2 cm. long and 0 5 cm wide, the cotton being thoroughly soaked, and then conveyed to the nasal mucous membrane, on each side, by gentle swabbing. In each series of experiments one or two volunteers were swabbed with the unheated filtrate and another, as a control, with the same material but heated at 60°C. for 1 hour. Furthermore, the cases were earlier in the course of the illness than those reported by Foster; namely, from 3 to 20 hours after the onset of the first symptoms.

Experiments on transmission from man to man were attempted with the filtered nasopharyngeal secretions from six patients in the early stages of infectious common colds. The following protocols are illustrative of the tests.

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[8] Flexner, S., and Noguchi, H., *J Exp Med.*, 1913, xviii, 461.

Protocol 1. Patient A.—Exposed to a case of a common cold 3 days previous to onset of symptoms. Illness characterized by a profuse, serous, irritating discharge from nose, slight conjunctivitis, but practically no fever or constitutional symptoms. This lasted for 2 days, after which the patient returned to normal. The nasopharyngeal secretions were collected in 25 cc. of Ringer's solution, 3 hours after the onset of the first signs of the affection. The unfiltered washings on aerobic culture showed growths of *Staphylococcus albus* and Pfeiffer's bacillus. The filtered washings on aerobic blood agar plate cultures yielded no growth. Anaerobic cultures of this and the following patients to be mentioned will be described later.

The filtrate was separated into two portions one, unheated, was applied by swabbing to the nasal mucosa of Volunteer 1, the other, heated at 60°C for 1 hour, to that of Volunteer 2. Volunteer 2 was unaffected.

Volunteer 1, in the afternoon of the 2nd day after swabbing, complained of malaise and dryness of the nose The next morning he presented a profuse, thin serous discharge from the nose, felt feverish, and suffered from headache. The following day the symptoms were aggravated, and the nasal discharge, which was profuse and mucoid, became purulent on the 4th day of the illness During the 5th to 7th days the affection subsided; the constitutional symptoms disappeared and only a slight purulent discharge was noted; but on the 8th day a right maxillary antrum infection supervened which endured for 2 weeks.

18 hours after the onset of the first symptom, the nasopharyngeal secretions of this person were collected in 25 cc of Ringer's solution The unfiltered washings showed on aerobic culture an occasional colony of *Staphylococcus albus* and *Streptococcus viridans*, the filtered washings yielded no growth.

The filtrate was treated as in the case of Patient A and applied, unheated, to the nasal mucosa of Volunteer 3 and heated on that of Volunteer 4 The latter was unaffected. Volunteer 3, who is ordinarily resistant to common colds, showed after 24 hours a profuse, thin discharge from the nose without any other symptoms. This lasted for only 1 day, after which the discharge disappeared.

Protocol 2 Patient B. Exposed to a case of a common cold 2 days previous to onset of symptoms There were no constitutional symptoms in this individual, only a profuse serous and later mucoid discharge from the nose and lacrimation, which endured for 2 days.

12 hours after the onset, the nasopharyngeal secretions were collected in 25 cc. of Ringer's solution The unfiltered secretions yielded an occasional colony of *Staphylococcus albus*, Pfeiffer's bacillus, and diphtheroids on aerobic culture; the filtered material exhibited no growth.

Volunteer 5 was swabbed nasally with the unheated filtrate, Volunteer 6 with the heated. The latter was not affected. Volunteer 5, 8 hours after swabbing, felt an increase of mucus in the nasopharynx On the next day there was a profuse, thin, serous secretion from the nasal mucosa but without constitutional disturbance. On the 2nd day the discharge became mucoid and the nose was clogged so that he was forced to breathe through the mouth. On the 3rd day the nasal secretions were profuse and purulent. The following day saw improvement in his condition, but on the 5th day there was an exacerbation of the acute attack. The duration of illness was 6 to 7 days.

20 hours after the onset of the affection the nasopharyngeal secretions of Volunteer 5 were collected in 30 cc. of Ringer's solution. The aerobic cultures of the unfiltered washings gave a few colonies of *Micrococcus flavus* and *Streptococcus viridans;* those of the filtrate were sterile.

The heated filtrate was swabbed on the nasal mucosa of Volunteer 7, who was not affected, and the unheated filtrate on that of Volunteer 8, who exhibited 8 hours later dryness of the nasal mucous membrane; 17 hours later there was an increased thin discharge without lacrimation or constitutional disturbance. 24 to 48 hours later the discharge was thicker, mucoid, and profuse, and on the 3rd day the subject was well.

Protocol 3. Patient C.—Two associates of this woman, including herself, became ill with common colds within 2 to 3 days of each other. 5 days after the onset of symptoms, her mother became ill with this disease. Patient C suffered from a mild attack; the 1st day was characterized by a profuse serous nasal discharge, the 2nd by a change in the nature of the secretions to mucoid and the 3rd day to mucopurulent. Except for lacrimation there were no symptoms.

6 hours after the onset, the nasopharyngeal secretions were washed with 30 cc. of Ringer's solution. The unfiltered washings yielded on aerobic culture numerous colonies of *Staphylococcus albus;* the filtered, no growth.

The filtrate was swabbed on the nasal mucosa of Volunteer 9. 14 hours later sneezing and dryness of nasal mucous membrane were noted. The next day this person showed injected conjunctivæ with lacrimation, sneezing and dryness of nasal mucosa, slight headache, and malaise. The following day there was a thin, serous discharge from the nose and slight laryngitis. The nasal symptoms and the laryngitis lasted for a day; thereafter the subject gradually returned to normal.

The nasopharyngeal washings from this volunteer were obtained 48 hours after the onset. The unfiltered material showed on aerobic culture a few colonies of *Micrococcus flavus* and *Streptococcus viridans;* similar tests on the filtrate were negative. The filtrate was swabbed on the nasal mucosa of Volunteer 10 who was not affected.

Protocol 4. Patient D.—This patient was in contact with two cases of common colds, one a week, the other 3 days previously. He had a profuse, thin, irritating, nasal discharge with excoriated skin about the nose. This condition endured for 3 days.

18 hours after the onset of symptoms, the nasopharyngeal secretions were collected by washing with 25 cc. of Ringer's solution. The unfiltered washings yielded a pure culture of aerobic diphtheroid bacilli; the filtered material was free from aerobic bacteria.

The heated filtrate was applied to the nasal mucosa of Volunteer 11, who remained normal. The unheated filtrate was swabbed on the nasal mucous membrane of Volunteer 12, who 24 hours later exhibited a profuse, mucoid, nasal discharge with collection of mucus in the nasopharynx. 48 hours later this person had a profuse, mucopurulent, nasal discharge and perspired freely (a symptom ordinarily concomitant with common colds in this man's experience). The symptoms disappeared 2 days later.

No attempts at further transmission were made in this instance.

Protocol 5. Patient E.—Contact with common cold case 2 days previous to onset of symptoms which were typical of those of a common cold. The nasopharynx was washed with 25 cc. of Ringer's solution 18 hours in the course of the affection. The unfiltered washings yielded Pfeiffer's bacillus, *Staphylococcus aureus*, and *Streptococcus viridans;* the filtered washings were free from aerobic bacteria.

The heated filtrate was swabbed on the nasal mucosa of Volunteer 12 and the unheated on that of Volunteers 13 and 14. None of these persons was affected.

Protocol 6. Patient F.—This patient was exposed to three others with common colds 3 days before the onset of his illness. The attack was typical and endured for 4 days. 20 hours after the onset of symptoms, the nasopharyngeal secretions were collected in 30 cc. of Ringer's solution. The unfiltered washings exhibited a few colonies of *Staphylococcus albus* and *Streptococcus viridans* on aerobic plate cultures; the filtered washings were free from growth.

The heated filtrate was applied to the nasal mucosa of Volunteer 15 and the unheated to that of Volunteers 16 and 17. All remained normal.

The experiments, although limited in number indicate the transmission of a condition similar to common colds from man to man with the filtered nasopharyngeal washings derived from four of six patients in the early hours of the disease. The two patients with whom transmission failed were 18 and 20 hours in the course of a common cold; the four from whom positive results were obtained were ill for 3, 6, 12, and 18 hours when the tests were made. In two of these four cases, transmission was effected to a second person; in one, such transmission failed, and in the fourth no attempt was made beyond the first passage. Altogether, therefore, definite symptoms were induced in six persons, of whom one developed a secondary maxillary antrum infection and the other a secondary laryngitis. Of the six persons, the first symptoms were noted 8 hours after swabbing in two cases, 24 hours in two, and 14 and 48 hours in the fifth and sixth individuals.

Control Experiments.

In the foregoing experiments it will be noted that the filtrates of the nasopharyngeal washings from early cases of common colds, heated for 1 hour at 60°C., failed to produce any effects in seven individuals. It seemed, however, that if parallel experiments should be made with the filtrates derived from cases regarded as suffering from non-infectious "colds," the evidence to be deduced from the control series would be strengthened.

Protocol 7. Patient G.—There was no contact with a case of a common cold but a definite history of exposure to cold and wet weather. Since then the patient had had a running nose without other manifestations, which lasted for 2 days.

12 hours after the onset, the nasopharyngeal secretions were washed with 25 cc. of Ringer's solution. The washings, which showed on aerobic culture *Streptococcus viridans* and Pfeiffer's bacillus, were filtered. The filtrate, free from aerobic bacteria, was swabbed on the nasal mucosa of Volunteer 18, who remained unaffected.

Protocol 8. Patient H.—There was no contact with a case of a common cold. Patient was exposed to continuous cold weather in an all day automobile ride. A thin discharge from the nose occurred on the next day and continued until the following noon. The nasopharyngeal secretions were collected in the first 3 to 4 hours after the onset in 25 cc. of Ringer's solution. Aerobic plate cultures yielded five colonies of *Staphylococcus aureus* from the unfiltered washings but no growth from the filtered material. The filtrate was swabbed on the nasal mucous membrane of Volunteer 19, who was not affected.

These two persons who evidently had non-infectious colds did not induce the transmission effects in inoculated individuals.

Cultivation Experiments.

We have already indicated, in the foregoing protocols, that cultures by ordinary means, such as by the employment of rabbit blood agar plates or veal infusion broth under aerobic conditions, did not yield growths of any constant or distinctively pathogenic microorganisms from either the nasopharyngeal washings or from rabbit lungs inoculated with these materials. The results with anaerobic methods will now be described.

The filtered nasopharyngeal secretions of 40 patients with early, typical, infectious common colds, and the unfiltered lung tissue suspensions of fifteen rabbits injected intratracheally with the secretions from ten of the patients, were cultured in the Smith-Noguchi, fresh tissue-ascitic fluid medium under a petrolatum seal. In none of the cultures was any predominating, pathogenic microorganism seen. It is noteworthy that in no instance was *Bacterium pneumosintes* found. In this regard, the nasopharyngeal secretions derived from common colds differ from those obtained in influenza, since this bacterium has been shown to bear a definite relationship to influenza.[1-8] We were also unable to cultivate the so-called "globoid bodies" of Foster.[7] The appearance of precipitate in this medium simulating morphologically definite bodies will be discussed below.

Later in the course of the cultivation experiments the anaerobic plate method was also used. This has already been described[1-8] and consists of 5 per cent rabbit blood plain agar in ordinary Petri dishes placed in the Brown anaerobic jar.[9] The advantages of the anaerobic plates in conjunction with the Smith-Noguchi fluid medium have been demonstrated in connection with the cultivation experiments with influenzal materials.[2]

The combined method of Smith-Noguchi medium and the anaerobic plates was employed in the cultivation of the filtered nasopharyngeal washings from nineteen of the forty cases mentioned. This procedure also failed to reveal a constant, definite, pathogenic agent, or Foster's globoid bodies. By means of the anaerobic plates, however, a number of microorganisms were isolated belonging to the three groups of anaerobic, filter-passing, Gram-negative bacteria described by Olitsky and Gates.[2]

Representatives of the first group of these microorganisms which are actively motile, spirochete-like, slender, curved bacilli, were obtained in eight of the nineteen cases studied. In two of the eight, bacteria of the other two groups were also found; in one, those of the second group and in the other case those of the third group. In a parallel series of experiments with six supposedly healthy individuals and six patients in the early hours of epidemic influenza, similar microorganisms were isolated in three of the normal controls and in two of the influenza cases. Of the latter two, in one instance bacteria of the third group were also obtained. In addition, this type of organism was recovered from a patient suffering from follicular tonsillitis.

The second group consists of minute, tenuous, pleomorphic bacilli. A representative of this group was obtained in one of the nineteen cases of common colds in the culture of which bacteria of Group I were also noted. The same species was cultivated from the nasopharyngeal secretions of one of two cases of acute follicular tonsillitis but not from those of nine supposedly healthy subjects or from the six cases of influenza.

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[9] Brown, J. H., *J. Exp. Med.*, 1921, xxxiii, 677; 1922, xxxv, 467.

The third group comprises bacteria of actively motile, very tenuous, slender, vibrio-like microorganisms which are smaller than those of the first group and are characterized by their failure to grow in fluid medium. Bacteria of this class were found in three of the nineteen cases of common colds, and in one of the three were associated with microorganisms of the first group. The same species was isolated in four of nine supposedly healthy individuals and in one of six cases of influenza.

Additional serological tests to those already reported[2] indicate that the rabbit immune serum produced by repeated injections of each type of these microorganisms shows each group to be distinct from the others; there is no cross-agglutination among the different types. Furthermore, these rabbit immune sera fail to agglutinate *Bacterium pneumosintes* and conversely, anti-*pneumosintes* immune serum does not agglutinate any of the microorganisms in the three groups.

Live cultures of fourteen strains of these "group" bacteria were injected intratracheally in rabbits to test their pathogenicity. None of the rabbits exhibited distinctive changes either in the blood or lungs or temperature. In only two instances were bacteria recovered from the lungs of the rabbits similar to those inoculated. Both of these microorganisms belonged to the first group. In addition to the rabbit tests for pathogenicity, a culture of each type was swabbed on the nasal mucosa of each of three persons. None was affected.

To sum up the cultivation experiments with the filtered nasopharyngeal secretions from early cases of typical infectious common colds, it appears that no single, constant, pathogenic agent was found in aerobic or anaerobic media. By means of a combination of Smith-Noguchi medium and anaerobic rabbit blood agar plates, however, a number of microorganisms belonging to the three groups already described by Olitsky and Gates[2] were isolated. Their irregularity of occurrence in common colds, influenza, and supposedly normal individuals, their differentiation into three distinct species, and their lack of pathogenicity for rabbits or man would indicate that they are not the incitants of common colds. Finally, it is to be emphasized that *Bacterium pneumosintes* was not found in any of the cultures from the nasopharyngeal secretions of common colds nor from those of the supposedly normal persons employed as controls.

DISCUSSION AND SUMMARY.

From the foregoing experiments it appears that with the filtered nasopharyngeal secretions from early cases of typical infectious common colds in the first 3 to 18 hours of the disease, a similar condition can be transmitted to man. With the unheated but not with the heated secretions from four of six such patients we have succeeded in transmitting an affection indistinguishable from common cold to four men and in two instances the condition was conveyed from the person with the experimental disease to a second individual—in all, therefore, to six supposedly normal subjects. The periods of incubation in the experimental disease varied from 8 to 48 hours. We failed to obtain these results with the filtered secretions from cases of common colds 18 and 20 hours after the onset of symptoms and from a patient with the experimental disease 20 hours after the first symptoms. It would appear that the secretions are more active in the early hours of the affection. We also failed in the two instances in which colds were caused by exposure to the elements, or chilling of the body, and not by definite contact with other cases of common colds.

Intratracheal inoculations in rabbits with unfiltered and filtered nasopharyngeal washings obtained from patients with common colds induce no characteristic or distinctive effects on the tissues, from which no constant, pathogenic agent has, as yet, been recovered. In comparison, similar material from cases of epidemic influenza do, however, cause particular changes in the blood and lungs of these animals, cultures of the lungs often yielding *Bacterium pneumosintes*.[1-3] In view of these facts and since the clinical pictures exhibited by these diseases differ, the conclusion may be drawn that infectious common colds and epidemic influenza are separate and distinct diseases. On the other hand, the negative results obtained with materials derived from common colds and from parallel series of experiments with secretions from supposedly healthy persons, serve as a control to the effects produced with the nasopharyngeal washings obtained from influenza patients.[1–3]

Aerobic and anaerobic cultures of the filtered nasopharyngeal washings from 40 early cases of infectious common colds have thus far yielded no constant, pathogenic agent which can be regarded as the incitant of the disease. The filtered washings of nineteen cases were studied by the combined method of Smith-Noguchi fluid medium and anaerobic blood agar plates. In these instances representatives of the three groups of anaerobic filter-passing, Gram-negative bacteria, described by Olitsky and Gates[2] were cultured from twelve patients. The irregularity of their occurrence not only in common colds but in influenza and supposedly normal persons and their lack of pathogenicity for rabbits and man indicate that these bacteria are not peculiar to common colds. This method has opened to view a number of hitherto undescribed microorganisms which can be found in different respiratory affections and in health. Furthermore, by morphological, cultural, and serological means, the separation into distinct species of each of these groups of bacteria has again been demonstrated. It is noteworthy that *Bacterium pneumosintes* was not found in any of the cultures from the 40 patients.

Special attention was given to the detection of elements similar to Foster's globoid bodies in the cultures derived from common colds and from the experimental disease in man, and from the lungs of inoculated rabbits. We have not been able to determine the presence of these bodies, although the precipitate which forms in fresh rabbit kidney tissue-ascitic fluid medium was illusory in such relation since it was a common experience to find this precipitate simulating the globoid bodies of poliomyelitis. Still more disturbing is the fact, that these particles could be carried over from subplant to subplant and even showed pseudo colony formation in the Noguchi semisolid medium in tubes.[8] But when the particles were put to rigorous test for a living and multiplying organism, the tests failed to reveal multiplication.

The increasing importance of the tissue-ascitic fluid medium in bacteriological technique warrants a detailed description of the requirements necessary for the determination of the living nature of formed elements in cultures in this medium. (*a*) No one method of staining can be relied upon, for stain precipitate in itself adds to the confusion. A specimen for examination should be stained separately by Gram's and Giemsa's methods and with another nuclear dye, such as polychrome methylene blue. As a rule, microorganisms will reveal their morphological characters in more than one stain, whereas precipitate may be found in only one and not in the others. The experienced eye will discern the precipitated particles in selected parts of the stained preparation where they often occur in enormous numbers, clumped into irregular masses of varying forms from the periphery of which there is a gradual fading out to finer, more uniform structures. (*b*) In addition, suspected growths should be tested in the dialysate medium of Gates[10] since by this method the precipitated material of the medium is kept from admixing with the growth, and a clear view of any microorganisms, if present, is obtained. (*c*) Another requirement is colony formation of the suspected culture. This is an absolute essential and can be effected by planting the material to be tested on solid plate media, incubated aerobically, and anaerobically in Brown's[9] jar. Semisolid medium in long tubes should also be employed but care is needed to avoid mistaking small particles of precipitate for actual colonies of bacteria. To make certain of growth of microorganisms in semisolid medium, however, subplanting to a precipitate-free, dialysate medium is required.

By following this method minute microorganisms which are obscured, or simulated, by precipitate in the Smith-Noguchi medium, can be identified.

CONCLUSIONS.

The transmission of a clinical condition similar to typical, infectious common cold from man to man with the filtered nasopharyngeal washings of early cases of the disease indicates that the incitant is filterable, thus confirming the earlier observations of Kruse and Foster.

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[10] Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 635.

Experiments on rabbits with these secretions and cultivation tests show that the materials derived from patients with common colds are distinct in effects from those of epidemic influenza.

Cultivations of the nasopharyngeal washings from 40 cases, and from the lung tissue of inoculated rabbits, have failed to reveal any constant, pathogenic agent, or incitant. Although a careful search was made for the "globoid bodies" of Foster in these materials, we have been unable to find them. None of these cultures, furthermore, yielded *Bacterium pneumosintes*.

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STUDIES CONCERNING THE INFLUENCE OF A DISTURBANCE IN THE ACID-BASE EQUILIBRIUM OF THE BLOOD ON RENAL FUNCTION AND PATHOLOGY.*

STUDY I. THE EFFECT OF ACID AND ALKALINE SOLUTIONS ON RENAL FUNCTION AND PATHOLOGY IN NORMAL DOGS.

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INTRODUCTION.

The following studies are not primarily concerned with the production of renal injuries by the use of acid and alkaline solutions in the sense of obtaining nephropathic processes which are comparable to injuries which may be induced experimentally in animals by various nephrotoxic substances, and which occur in man from a variety of causes. The main interest in the different series of experiments has been to observe the functional and pathological response of the kidneys when these organs were furnished a blood of altered chemical composition by the introduction of an acid or an alkaline solution.

The ability of cells to functionate in a normal manner is profoundly influenced by the proper balance between hydrogen and hydroxyl ions in the fluid which bathes the cells and which gives to them their ever changing physico-chemical environment.

L. J. Henderson[1] in one of his numerous contributions to this field of investigation has stated so clearly the importance of the physico-chemical environment of cells that I take the liberty of quoting one of his statements. "The right working of physiological processes depends upon an accurate adjustment and preservation of physico-chemical conditions within the body. Throughout the

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animal body, while life exists, there occurs a regular formation of acid substances, excretory products of metabolism. Through resulting changes in equilibria between bases and acids, normal metabolism steadily operates to lower the invarying alkaline (neutral) reaction of the body. Like temperature and osmotic pressure, the neutrality or alkalinity is adjusted by a mechanism within the body, but permanently maintained by exchanges with the environment."

In the studies which are to follow, the kidney has been selected as the organ in which to observe the influence of changes in this acid-base balance of the blood on its function and pathology for two reasons. In the first place the product of the kidney's functional response can be easily obtained and its composition determined. The kidney, unlike such organs as the liver, lends itself to a variety of fairly accurate functional tests which can be easily performed. Secondly; one of the essential functions of the kidney in health, as well as under the strain of certain diseased states, is to maintain either by elimination or retention of hydrogen and hydroxyl ions such a balanced neutrality of the blood that the kidney as well as other groups of cells may have a proper physico-chemical environment in which to functionate in a normal manner. For this latter reason in particular, it is of interest to study the functional and pathological response of the normal and previously diseased kidney when that physico-chemical balance of the blood which it so largely maintains at a point of neutrality is disturbed by the introduction of an acid or an alkaline solution.

The following investigation is divided into three parts. The first study is concerned with the influence of acid and alkaline solutions on renal function and pathology in normal animals. The second part deals with the influence of acid and alkaline solutions of the same strength on renal function and pathology in naturally nephropathic animals. The third part of the study is concerned with the ability of an alkaline solution to protect both the normal and naturally nephropathic kidney against injury from an acid solution.

The literature dealing with the effect of acids and alkalies when introduced into organisms has been mainly concerned with the metabolic disturbance induced by such injections, and with the way in which the disturbance which takes place in the acid-base equilibrium of the blood is readjusted. Only incidental attention has been paid to the influence of such solutions on renal function and pathology.

In a study concerning the alkalescence of the blood, Lassar[2] observed, by crude methods of determination, a decrease in the alkalinity of the blood in dogs that had received an acid intravenously. In 1877, Walter,[3] in a research concerning the action of acids on the animal organism, made the observation that in animals killed by the injection of acid into the blood, the blood apparently remained alkaline. He noted a depletion in the bases of the blood as shown by a decrease in the carbon dioxide tension, and furthermore made the observation of an increase in ammonia in the urine. The animals succumbed from a respiratory type of death which could be delayed by the use of sodium carbonate.

Loeb[4] in 1898 pointed out that whenever oxidations are impaired in tissues, the osmotic pressure rises, and ascribes this to an accumulation of incompletely oxidized metabolic products, particularly acids. He observed that under such conditions muscle tissue took up water and became edematous. In later publications[5,6] Loeb has shown the importance of carbonates in the maintenance of the neutrality of tissue fluids and the media surrounding living cells.

Hoppe-Seyler and Araki,[7] in one of the early and important papers on cell oxidations, made the observation that an interference with such processes leads to the abnormal production and accumulation of acids.

In the same year Hofmeister[8] demonstrated the influence on colloidal swelling of variations in the concentration of hydrogen and hydroxyl ions.

Some years later than these observations the studies of Fischer[9,10] on edema and nephritis provoked much clinical interest and led to such critical analyses of the importance of a disturbance in the acid-base equilibrium of the organism as a factor in inducing edema and nephritis as are found in the study by Henderson, Palmer and Newburgh.[11]

The more recent literature has been concerned with the effect of acid and alkaline solutions on metabolism and to a less extent with the ability of such changes in the blood as these solutions induce to injure the kidney.

In 1918 Bornstein and Lippmann[12] suggested that the products of vigorous metabolism occurring in swimmers and in men subjected to heavy marching were a cause of certain transitory albuminurias. They noted a striking parallelism between the output of albumin and the presence of cylindroids in the urine with the acidity of the urine. The presence of both albumin and cylindroids was checked by the use of an alkali.

Fitz, Alsberg and Henderson,[13] in a study of the excretion of phosphoric acid during an experimental acidosis in rabbits induced by giving hydrochloric acid, demonstrated that phosphates, as the mono and dipotassium phosphates, constituted a nearly neutral solution which had the property of taking up large quantities of acid or alkali. They concluded that slight changes in hydrogen ionization can hardly be without influence on the catalytic reactions of protoplasm.

Nagayama,[14] in his studies on renal activity and the acid-base equilibrium, observed the urea-excreting activity of the kidney to be less after administering an acid phosphate than after a neutral phosphate of the same phosphorus content. He concludes that the decrease in the alkalinity of the plasma decreases the urea-excreting functionof the kidney. Alkaline phosphates in equivalent amount to the acid phosphate had no appreciable effect.

The influence of acid and alkaline solutions has been studied in even more fundamental relations than their effect on metabolism and renal function. Jewell[15] in a study on the effect of hydrogen ion concentration and the oxygen content of water upon tadpoles, observed the optimum hydrogen ion concentration to be approximate neutrality. Variations from this in either direction, as well as low temperature and low oxygen content of the water, progressively decreased both the rate and total amount of regeneration in tadpoles.

In two studies[16,17] from this laboratory, it has been shown that the toxicity of uranium nitrate, which induces in part its injurious effect through the establishment of a disturbance in the acid-base equilibrium of the blood, is in large measure dependent upon the age of the organism in which a given degree of disturbance in this balance is induced. The tissues of old animals are more susceptible to such a disturbance than are the tissues of young animals. At a later date McArthur[18] confirmed these observations by demonstrating in planarians a decrease in the tolerance to acid and alkaline solutions as the age of the organism increased.

The following investigations have been undertaken to study the functional and pathological response not of the organism as a whole, but of an easily accessible organ, namely the kidney, to a disturbance in its physico-chemical environment induced by the introduction of acid and alkaline solutions into the blood stream.

STUDY 1.

The Effect of Acid and Alkaline Solutions on Renal Function and Pathology in Normal Dogs.

TECHNIQUE OF EXPERIMENTS.

Thirty-eight normal dogs were used in this series of experiments. Eighteen of these animals were used in the first part of the study and were given intravenously a solution of hydrochloric acid. Six of the animals were employed as controls and were not subjected to the action of the acid solution. The remaining eighteen animals of the series were used in the second part of the investigation and, with the exception of six animals that were reserved for control experiments, were given intravenously an alkaline solution.

The observations on these animals prior to any experimental interference has been similar to that described in previous publications. The dogs were kept in metabolism cages for four days before the day of experiment and were fed on lean raw beef and bread made from corn meal. Two hundred and fifty c.c. of water was given twice a day by stomach tube. The urine was collected twice a day and subjected to the routine qualitative analysis for albumin, glucose and diacetic acid. Microscopic examinations were made each day from catheterized specimens. Two days before the day of experiment the functional response of the kidney was determined by the phenolsulphonephthalein test as conducted by Rowntree and Geraghty.[19] The elimination of the dye was estimated for a two hour period. For four days prior to the experiments the reserve alkali of the blood was determined by the method of Marriott.[20]

The technique employed in the experiments was as follows: Three hours before commencing an experiment the animal was given 250 c.c. of water by stomach tube. At the end of this period the animals were anesthetized by Gréhant's* anesthetic in 60 per cent. strength. An hour was allowed for the development of a satisfactory state of anesthesia to permit the necessary operative interference. The animals were placed in a wooden operating rack which fits into the concavity of a copper box containing hot water. By such a device which was first employed in Sollmann's laboratory, an attempt was made to maintain the animal's normal body temperature during the period of the experiment. A tracheal canula was introduced for use in case it became necessary to employ either during the anesthesia. The carotid artery was exposed, a canula tied in place and connected in the usual manner to a mercury manometer. The femoral vein was exposed, a canula tied in place and connected with a burette. Through this connection the acid or alkaline solutions were introduced into the animals. The abdomen was opened through a small midline incision and canulas tied into each ureter. Urine flow was determined in drops per minute.

During the course of the experiments samples of blood were obtained from either the unused femoral vein or the external jugular veins for making determinations of the alkali reserve of the blood.

At the conclusion of the operative part of the experiments all of the animals were given intravenously 25 c.c. per kilogram of a warm 0.9 per cent. sodium chloride solution with the object in view of producing a free flow of urine.

NORMAL DOGS.

The Effect of a N/2 Solution of Hydrochloric Acid on Renal Function and Pathology.

Eighteen normal dogs were used in this series of experiments. Six of the dogs were used for control experiments. These animals received the intravenous injection of isotonic sodium chloride solution but were not given the acid solution. The experiments lasted over a four hour period. For the first two hours of this period observations were made on carotid blood pressure, urine flow and the reserve alkali of the blood at half hour intervals. For the last two hours of the experiments these observations were made at hour intervals.

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* Gréhant's Anesthetic. The animal is given 0.25 c. c. per kilogram of a 4 per cent. solution of morphine sulphate. This is followed in half an hour by 10 c. c. per kilogram of the following mixture: Chloroform, 50 c. c.; alcohol and water, each 500 c. c.

The urine formed by the animals during the course of the experiments was collected at the end of the first two hour period and examined for albumin, casts and diacetic acid. At this stage of the experiment 1 c.c. of the usual solution of phenolsulphonephthalein was injected subcutaneously, the urine collected for the remaining two hour period, and the quantitative output of the dye determined.

At the end of the experiments the kidneys were removed and the tissue fixed in a corrosive acetic solution, Zenker's fluid, 95 per cent, alcohol, and in a 10 per cent. formaline solution. Sections from this tissue were stained with haematoxylin and eosin and with eosin and methylene blue. Fresh kidney tissue was frozen and sections made. Such sections were stained for lipoid material by Herxheimer's Scharlach R method. The deductions which will be made concerning the pathology of the kidney in the various series of experiments are based on a histological study of such sections.

Immediately following the initial observations on urine flow and blood pressure, the animals of this series other than the animals of the six control experiments, were given intravenously 5 c. c. per kilogram of a N/2 solution of hydrochloric acid. At the end of the first hour of the experiments, the injection was repeated. The results obtained from such injections as contrasted with the control experiments will be found in Table 1, Study 1.

A study of this table shows that all of the animals prior to the commencement of the experiments were normal, in so far as renal function and a normal alkali reserve of the blood were concerned. The urine was free from albumin, glucose and diacetic acid. Tube casts were not present. The elimination of phenolsulphonephthalein by the respective animals in a two hour period varied from a minimum output of 58 to a maximum output of 84 per cent. The reserve alkali of the blood in the different animals varied from 8.0 to 8.1.

Control Experiments.

Six normal dogs have been used for control experiments to ascertain the effect of Gréhant's anesthetic on renal function and the acid-base equilibrium of the blood during an experimental period of four hours and to note the changes in the kidney at the end of such a period of anesthesia. The results obtained in four of these animals, Experiments 1, 4, 7 and 10, are included in Table 1.

Immediately following the development of a satisfactory state of anesthesia the animals were given intravenously 25 c. c. per kilogram of 0.9 per cent. sodium chloride solution. Following the use of the solution of sodium chloride all of the animals developed a fair state of diuresis. The flow of urine in the respective animals varied from 8 to 16 drops per minute. The systolic blood pressure in the different animals varied from a minimum of 112 mm. of mercury in the animal of Experiment 4, to a maximum of 138 mm. of mercury in the animal of Experiment 10.

At the end of the first hour period of the experiments the reserve alkali of the blood remained unchanged from the normal readings obtained prior to the use of the anesthetic. At this stage there had developed a slight reduction in the systolic blood pressure of all of the animals. The flow of urine in the animal of Experiment 10 showed an increase from 8 to 13 drops per minute. The urine flow in all of the other control animals had undergone a slight reduction. In the animal of Experiment 1, urine flow was reduced from 12 to 9 drops per minute, in Experiment 4, from 16 to 12 drops per minute, and in Experiment 7, from 10 to 7 drops per minute.

At the end of the second hour of the experiments but little change had taken place in the general condition of the animals or in the functional response of the kidneys.

The reserve alkali of the blood in all of the animals, except the animal of Experiment 7, had undergone a slight depletion. In the animal of Experiment 1, the reserve alkali was reduced from the normal reading of 8.1 to 8.0, in Experiment 4, from 8.0 to 7.95, and in Experiment 10, from 8.1 to 8.0.

All of the animals remained diuretic. The urine flow in the animal of Experiment 1 had increased from 10 to 16 drops per minute, and in Experiment 4 the animal showed an increase in urine formation from 8 to 17 drops per minute. In the remaining animals the flow of urine was reduced. In the animal of Experiment 7, the flow of urine was reduced from 10 to 8 drops and in the animal of Experiment 10, from 6 to 4 drops per minute.

At this stage of the experiments the systolic blood pressure of the respective animals showed but slight change from the normal readings. The lowest blood pressure was 106 mm. of mercury and the highest pressure was 127 mm. of mercury.

At the termination of the experiments, four hours after the initial observations, all of these control animals showed a reduction in urine formation. The flow of urine in the animal of Experiment 4 was reduced from 17 to 12 drops per minute, while in the animal of Experiment 10 urine formation had been reduced from 6 drops to 1 drop per minute.

The reserve alkali of the blood at the end of the four hour period not only failed to show a further depletion from that noted at the end of the second hour of the experiments, but in two of the animals there had developed during the last two hours of the period an attempt to restore the normal acid-base equilibrium of the blood. In the animal of Experiment 1, the reserve alkali of the blood had increased during the latter half of the experimental period from 8.0 to 8.1. In the animal in Experiment 4, an increase had taken place from 7.95 to 8.0.

It is interesting to note that in these animals urine formation was in excess of the other control animals which during the period of anesthesia not only showed a reduction in the reserve alkali of the blood but also showed an inability to readjust the acid-base equilibrium of the blood.

During the period of anesthesia the systolic blood pressure in all of the animals showed some reduction. This was most marked in the animal of Experiment 10, STUDY I.

Normal Dogs. The Effect of a N/2 Solution of

Number of Experiment	Urine	Phthalein 2 hour period	R pH	Gréhant's Anesthetic 60%	Urine per minute Bl. Pres. mm. Hg	Acid or Alkali	R pH end of 1st half hour	Urine per minute Bl. Pres. mm. Hg	R.pH end of 2nd half hour	Urine per minute Bl Pres. mm. Hg
1 Control	Normal	77%	8 1	25 cc. per kg. 0 9% NaCl	Urine 12 B. P. 123	0	8 1	Urine 14 B P. 120	8 1	Urine 9 B. P 120
4 Control	Normal	65%	8 0	25 cc per kg. 0 9% NaCl	Urine 16 B. P. 112	0	8 0	Urine 14 B. P 117	8 0	Urine 12 B P. 108
7 Control	Normal	74%	8 1	25 cc per kg. 0 9% NaCl	Urine 10 B. P 126	0	8 1	Urine 12 B P 131	8 1	Urine 7 B. P 112
10 Control	Normal	82%	8 1	25 cc per kg 0 9% NaCl	Urine 8 B. P 138	0	8 1	Urine 9 B P 130	8 1	Urine 13 B. P 124
5	Normal	84%	8 1	25 cc per kg 0 9% NaCl	Urine 11 B P 112	5 cc per kg N/2 HCl	7 9	Urine 27 B P 131	8 05	Urine 31 B P 130
6	Normal	68%	8 0	25 cc per kg 0 9% NaCl	Urine 9 B P 131	5 cc per kg N/2 HCl	7 9	Urine 14 B P. 136	7 95	Urine 16 B P 128
8	Normal	70%	8 0	25 cc per kg 0 9% NaCl	Urine 12 B P. 118	5 cc per kg. N/2 HCl	7 9	Urine 22 B P 124	7 9	Urine 20 B P 124
12	Normal	67%	8 1	25 cc per kg. 0 9% NaCl	Urine 6 B P. 124	5 cc per kg N/2 HCl	7 8	Urine 18 B. P 116	7 95	Urine 10 B P 120
13	Normal	58%	8 0	25 cc per kg 0 9% NaCl	Urine 4 B. P. 114	5 cc per kg. N/2 HCl	7 9	Urine 26 B P 118	8 0	Urine 18 B P 108
14	Normal	60%	8 0	25 cc per kg 0 9% NaCl	Urine 13 B. P. 100	5 cc per kg N/2 HCl	7 9	Urine 36 B P 110	7 95	Urine 14 B P. 100
16	Normal	74%	8 0	25 cc per kg. 0 9% NaCl	Urine 16 B. P. 126	5 cc per kg N/2 HCl	7 75	Urine 48 B P 132	7 9	Urine 20 B. P 108
18	Normal	58%	8 1	25 cc per kg 0 9% NaCl	Urine 7 B P. 135	5 cc. per kg N/2 HCl	7 9	Urine 30 B. P 140	8 0	Urine 18 B. P 140

in which a reduction occurred from the normal of 138 mm. of mercury to 110 mm. of mercury.

At the commencement of the last two hours of the experiments all of the animals were given subcutaneously 1 c. c. of a solution of phenolsulphonephthalein for a renal function test. The elimination of the dye was slightly reduced in all of the animals. The most marked reduction was in the animal of Experiment 10. The normal elimination for this animal before the experiment was 82 per cent. During

TABLE 1.

Hydrochloric Acid on Renal Function and Pathology.

Acid or Alkali	R pH end of 3rd half hour	Urine per minute Bl. Pres mm Hg	R.pH end of 4th half hour	Urine per minute Bl. Pres. mm Hg	R.pH end of 3rd hour	Urine per minute Bl. Pres. mm Hg	R pH end of 4th hour	Urine per minute Bl Pres. mm Hg	Phthalein 2 hour period	Albumin and Casts	Diacetic acid
0	8 1	Urine 10 B. P. 118	8 0	Urine 16 B. P. 127	8 0	Urine 8 B P. 116	8 1	Urine 8 B. P. 120	65%	0	0
0	7 95	Urine 8 B P 112	7 95	Urine 17 B. P. 106	7 95	Urine 12 B. P. 111	8 0	Urine 11 B. P. 105	50%	0	0
0	8 1	Urine 10 B. P. 116	8 1	Urine 8 B. P. 120	8.05	Urine 5 B P. 122	8 05	Urine 4 B P. 108	66%	Trace of albumin No casts	0
0	8 1	Urine 6 B P 118	8 0	Urine 4 B. P. 116	8 0	Urine 3 B. P. 120	8 0	Urine 1 B. P. 110	65%	0	0
5 cc per kg N/2 HCl	7 9	Urine 16 B. P. 128	7 9	Urine 8 B P 133	7 9	Urine 4 B P. 127	7 95	Urine 5 B P. 120	40%	Present	Present
5 cc per kg. N/2 HCl	7 8	Urine 7 B. P 120	7 85	Urine 2 B P 125	7 9	Urine 0 B. P. 131	7 9	Urine 2 B. P. 118	50%	Heavy trace Casts	Present
5 cc per kg N/2 HCl	7 8	Urine 18 B P 127	7 9	Urine 11 B P. 120	7 85	Urine 4 B P 110	7.95	Urine 1 B P. 103	25%	Heavy trace Casts	0
5 cc per kg. N/2 HCl	7 8	Urine 10 B P 105	7 8	Urine 10 B P. 108	7 9	Urine 4 B. P 110	7 9	Urine 4 B. P. 114	30%	Heavy trace Casts	Present
5 cc per kg N/2 HCl	7 85	Urine 10 B P 118	7 85	Urine 5 B. P 108	7 9	Urine 2 B. P. 110	7 9	Urine 2 B P. 112	20%	Heavy trace Casts	Present
5 cc per kg N/2 HCl	7 8	Urine 4 B P 108	7 95	Urine 1 B P 108	7 95	Urine 1 B P. 100	7 95	Urine 1 in 2 minutes B P 91	35%	Very heavy trace Casts	0
5 cc per kg N/2 HCl	7 75	Urine 20 B P. 112	7 75	Urine 8 B P. 110	7 8	Urine 5 B P. 105	7.9	Urine 6 B P. 100	50%	Very heavy trace Casts	Present
5 cc per kg N/2 HCl	7 85	Urine 12 B. P 140	7 9	Urine 10 B. P. 130	7 95	Urine 10 B P 120	7 95	Urine 8 B P. 125	50%	Faint trace No casts	Present

the last two hours of the experimental period the output was 65 per cent. The eliminations of the dye by the other animals of the control series varied from a minimum output of 50 per cent. to a maximum output of 66 per cent.

Urine collected during the course of the control experiments with one exception was free from albumin and diacetic acid. The urine from the animal of Experiment 7 contained a trace of albumin but no casts.

The histological study of the kidneys from the control group of animals has shown but slight evidence of injury. The amount of stainable lipoid material is increased in the cells of the loops of Henle over that found in normal unanesthetized dogs. There occurs a moderate grade of cloudy swelling in the epithelium of some of the convoluted tubules. These cells have failed to show vacuolation or necrosis. Fig. 1, Study 1.

Conclusions Concerning the Control Group of Animals.

1. Gréhant's anesthetic induces a satisfactory state of surgical anesthesia in normal dogs for a period of four hours.
2. During such a period of anesthesia there occurs in all of the animals some reduction in blood pressure. The maximum reduction has been 28 mm. of mercury.
3. All of the animals have remained diuretic during the course of the experiments but the flow of urine per minute has been reduced.
4. The elimination of phenolsulphonephthalein is reduced. The maximum output of the dye in a two hour period has been 66 per cent. and the minimum output 50 per cent.
5. With one exception the urine from all of the control animals has been free from albumin, casts and diacetic acid.
6. Three of the six control animals showed no reduction in the alkali reserve of the blood during the experimental period while the three remaining animals showed a depletion. Two of these animals during the last two hours of the experiments were able to re-establish a normal acid-base equilibrium of the blood to the reading obtained before the commencement of the experiments. Urine formation by these animals was in excess of the amount formed by those animals in which a re-establishment of the acid-base equilibrium of the blood was not effected. The animal of the series (Experiment 10) in which the most marked depletion in the alkali reserve of the blood developed was the least diuretic member of the group and the elimination of phenolsulphonephthalein by this animal showed a greater reduction than was shown by any other member of the series of control animals.

The observation of outstanding interest in connection with this group of animals is that after the early stages of the experiments when a depletion in the alkali reserve of the blood has been induced, such normal animals may, as the duration of the experiments is prolonged, effect a re-establishment of this depletion. Associated with such a restoration in the blood chemical environment of the kidney urine formation and phenolsulphonephthalein elimination are favored.

The Effect of a N/2 Solution of Hydrochloric Acid on Renal Function and Pathology in Normal Dogs.

Twelve animals were used in this series of experiments. The experiments were conducted in an identical manner with the previously described control group, with the exception that after the initial observations had been made the animals were given intravenously 5 c. c. per kilogram of a N/2 solution of hydrochloric acid. This injection was repeated at the end of the first hour of each experiment.

Eight representative experiments are included in Table 1, Study 1.

Studies made of the animals before the experiments showed them to be normal. The urine was free from albumin, glucose and diacetic acid. The elimination of phenolsulphonephthalein by the respective animals varied from 58 to 84 per cent. The reserve alkali of the blood varied from 8.0 to 8.1.

Following the development of a state of anesthesia and the routine operative interference the dogs were given intravenously the usual injection of isotonic sodium chloride solution. A fair degree of diuresis ensued. The flow of urine by the different animals varied from 4 to 16 drops per minute.

The systemic blood pressure varied from 100 mm. to 135 mm. of mercury.

At this stage of the experiments the animals were given intravenously the solution of hydrochloric acid. Such injections induced a fast, followed by a slow and deep type of respiration which gradually became normal.

The immediate effect of such an injection was to reduce rapidly the reserve alkali of the blood in all of the animals. The maximum reduction from the normal of 8.0 to 7.75 developed in the animal of Experiment 16. In the animal of Experiment 12, the reserve alkali was reduced from 8.1 to 7.8, while in the remaining animals no reduction occurred below 7.9.

The result of such an injection in normal animals early in the experimental period is very constant. By the end of the second half hour period all of the animals had become markedly diuretic, and with one exception, they had either restored their normal acid-base equilibrium or they were in the process of such a restoration. For example: the animal of Experiment 16 had a normal alkali reserve of the blood of 8.0. Following the acid injection this was reduced to 7.75. The urine flow increased from 16 to 48 drops per minute and within an hour the reserve alkali had risen to 7.9. The animal of Experiment 13 had a normal alkali reserve of 8.0. Following the use of the acid solution the reserve alkali was reduced to 7.9. The flow of urine increased from 4 to 26 drops per minute and at the end of the first hour period the alkali reserve had been restored to the normal reading of 8.0.

During this early period of the experiments very slight changes developed in the systolic blood pressure of the animals. The blood pressure for the different animals varied from 100 to 140 mm. of mercury.

At the end of the second half hour period of the experiments the second and final injection of 5 c. c. per kilogram of a N/2 solution of hydrochloric acid was given. At this period of the experiments all of the animals were more freely diuretic than they were before the first acid solution was administered. The reserve alkali of the blood had either been restored to the normal or it had increased from the reduction induced by the first acid injection.

The second intravenous injection of the acid was followed by a disturbance in the breathing such as been referred to and by an associated and abrupt reduction in the reserve alkali of the blood. The maximum reduction occurred in the animal of Experiment 16, in which the alkali reserve was reduced to the same reading as was obtained from the first acid injection 7.75. The least reduction occurred in the animal of Experiment 5, in which the reserve alkali was depleted from 8.05 to 7.9. The remaining animals showed an alkali reserve which varied from 7.8 to 7.85.

The reductions in the alkali reserve of the blood from the second injection of the acid solution were in general greater than from the first injection. The effect on urine formation and the rapidity with which an attempt is made to restore the acid-base equilibrium of the blood following the second injection of such a solution is strikingly different from that obtained when the first injection of an acid solution was used.

Following the second injection of the hydrochloric acid solution there was no increase in urine formation by any of the animals. With two exceptions urine formation was reduced, while in Experiments 12 and 16 no change occurred in the formation of urine. By the end of the first hour following the second dose of the hydrochloric acid solution a further reduction in urine formation had developed so that the maximum formation of urine by any animal of the series, Experiment 8, was 11 drops per minute, while the minimum output by the animal of Experiment 14, was 1 drop per minute.

The respective animals at this stage of the experiments manifest an inability to restore the acid-base equilibrium of the blood, and associated with this change in the physico-chemical state of the blood which is furnished the kidney there is a reduction in urine formation. For example, following the first injection of the acid solution in the animal of Experiment 16, the reserve alkali of the blood was reduced to 7.75, and within an hour it was restored to 7.9. The second injection of the acid solution in this animal reduced the alkali reserve to the same reading, 7.75, and at the end of an hour no change had taken place in the reading. No increase in urine formation occurred. In the animal of Experiment 13, the reserve alkali of the blood was reduced by the first acid solution from the normal of 8.0 to 7.9. At the end of an hour this reading had returned to the normal and the kidney was functionally active. Following the second injection of the acid solution the reserve alkali was reduced from 8.0 to 7.85, and at the expiration of an hour no change had taken place in the degree of depletion. Urine formation was decreased.

The response of these normal animals to a second injection of hydrochloric acid solution differed from the initial response of the animals in that in general a greater reduction in the alkali reserve of the blood develops; the animals do not become diuretic but on the contrary usually show a reduction in urine formation; and finally there is either no attempt on the part of the animals to restore the acid-base equilibrium of the blood in an hour period or the restoration is delayed as compared with the response of the animals to the first injection of such an acid solution.

During the remaining two hours of the experiments there occurred a gradual reduction in urine formation. At the end of the experiments the maximum output of urine by the animal of Experiment 18 was 8 drops per minute. The minimum output by the animal of Experiment 14, was 1 drop every two minutes.

During the last two hours of the experiments the reserve alkali of the blood showed a gradual increase but in none of the animals was the normal reserve alkali re-established. These readings for the respective animals varied from 7.9 to 7.95.

The systolic blood pressure in the different animals has been well maintained throughout the experiments and has varied from 91 mm. of mercury to 125 mm. of mercury.

The effect of the acid solutions on renal function are clearly shown by the changes in the urine and by the elimination of phenolsulphonephthalein. The urine of all of the animals showed the presence of albumin. This varied from a faint trace to a heavy precipitate. Both hyalin and granular casts were present in the urine of eight of the twelve dogs. Diacetic acid was present in the urine of nine of the animals.

All of the animals showed a decided reduction in the elimination of phenolsulphonephthalein. The normal elimination of the dye by the animal of Experiment 5, was 84 per cent. As a result of the use of the acid solutions the elimination was reduced to 40 per cent. The animal of Experiment 13 had a normal elimination of 58 per cent. of the dye. Following the experiment this was reduced to 20 per cent.

The histology of the kidneys of these normal animals that have received two intravenous injections of N/2 hydrochloric acid shows the following changes. The amount of stainable lipoid material appearing in the loops of Henle is very greatly increased over that which could be demonstrated in the same location in the control group of animals. Furthermore, such stainable material appears as droplets in cells of the convoluted tubules. The epithelium of the tubules, and especially that of the convoluted tubules, shows advanced cloudy swelling and the accumulation of granulas throughout the cytoplasm. The nuclei frequently stain imperfectly. The cells have but rarely shown vacuolation and necrosis of the epithelium has not been observed. Fig. 2, Study 1.

Conclusions Concerning the Group of Normal Animals That Received a N/2 Solution of Hydrochloric Acid.

1. Following the intravenous administration of 25 c.c. per kilogram of an isotonic solution of sodium chloride to normal dogs, there has developed in eight of the twelve animals receiving such a solution a moderate grade of diuresis. The flow of urine in the different animals has varied from 4 to 16 drops per minute. In four normal animals that received such injections there was no increase in urine formation. The introduction of such an amount of fluid increases the volume of blood, induces a temporary hydremic state of the blood, and causes a transitory rise in blood pressure. With such conditions favorable for urine formation, the kidneys respond to this normal type of solution by either no increase in urine formation or by an increase which does not exceed the maximum output of 16 drops of urine per minute.

2. When these normal dogs, early in an experiment, before any damage has been induced in the kidneys, are given a solution of 5 c.c. per kilogram of a N/2 solution of hydrochloric acid intravenously, the solution being essentially not a normal solution, a change is induced in the physico-chemical state of the blood which at once throws into operation a mechanism in the kidney in an attempt to re-establish a normal blood chemical environment for the organism and for its own functional response. The use of such an acid solution at this early stage of the experiments caused a sudden reduction in the reserve alkali of the blood and a profuse diuresis. The greatest reduction in the reserve alkali of the blood was from a normal of 8.0 to 7.5. The most marked increase in urine formation occurred in the same animal. The flow of urine increased from 16 to 48 drops per minute.

Following the marked diuretic effect of such an acid solution in an uninjured kidney, the animals have within an hour either established a normal acid-base equilibrium of the blood or such an increase has occurred in the reserve alkali of the blood that the readings approach the normal.

There is a marked difference in the response of the kidney to these two types of solutions. The response of the normal kidney to an isotonic solution of sodium chloride, which does not deplete the reserve alkali of the blood, is either a negative response, or the diuretic effect is slight and not commensurate with the volume of fluid administered intravenously. The response of the normal kidney to an acid solution of less volume, but an essentially abnormal solution of such a composition that it induces a marked reduction in the alkali reserve of the blood, is a profuse diuresis, and associated with this functional response there occurs a partial or complete restoration of the acid-base equilibrium of the blood. As this equlibrium is attained there develops a decrease in urine formation.

3. A study of the urine formed by the different animals of the series at this stage of the experiments; viz., the end of the first hour, has shown the presence of albumin, and in the majority of the animals casts have also been present. It would appear that, even though the normal kidney could respond to the changed physico-chemical state of the blood induced by the acid solution and in part re-establish a normal environment for the organism, an injury was done to the functional unit, the kidney, which in large measure effected such a readjustment.

4. Following the second injection of the acid solution, the evidence of the renal injury induced by the first injection of such a solution is seen by the lack of response of the kidney to the second injection even though the reserve alkali of the blood in the various animals was depleted to a greater extent than was the case from the first injection. With an even greater demand for the restoration by the kidney of a departure from the normal in one of the fundamental physico-chemical states of the blood, this injured functional unit is unable to make the necessary response. The second injection of the acid solution increased the flow of urine in only one animal of the series. In the other animals the flow of urine was either reduced or it remained unaffected. During the remaining three hours of the experiments the reserve alkali of the blood was gradually increased but in none of the animals was the normal acid-base equilibrium of the blood attained.

The Effect of Alkaline Solutions of Different Molecular Concentration on Renal Function and Pathology in Nornal Dogs.

Eighteen dogs were used in this series of experiments. The animals were subjected to an experimental technique identical with that outlined for the animals used in the first part of this study. The urine from all of these animals before the commencement of the experiments was normal. It did not contain albumin, casts, glucose, or diacetic acid. The elimination of phenolsulphonephthalein by the respective animals in a two hour period varied from a minimum output of 60 per cent. to a maximum output of 80 per cent. The reserve alkali of the blood was normal and varied from 8.0 to 8.1.

At the completion of the anesthesia with Gréhant's anesthetic the animals were were given intravenously the usual preliminary injection of 25 c. c. per kilogram of 0.9 per cent. sodium chloride solution. Observations on urine flow, the reserve alkali of the blood, and systolic blood pressure were made at the usual intervals during the experiments. Eight of the animals were given intravenously 25 c.c. per kilogram of a solution of sodium carbonate equimolecular with a 1.5 per cent. solution of sodium chloride, while the remaining ten animals were given a similar amount per kilogram of a solution of sodium carbonate equimolecular with a 3 per cent. solution of sodium chloride.

The following studies have been made on the ability of normal dogs to readjust the physico-chemical state of the blood, when this environment has departed from the normal by the introduction of alkaline solutions of the concentrations above mentioned. Associated with such a study observations have been made on the functional and pathological response of the kidney when the blood chemical environment of this organ is made to depart from the normal by the use of such solutions. Twelve experiments representative of the results obtained in this group of animals are incorporated in Table 2, Study 1.

The Effect of a Solution of Sodium Carbonate Equimolecular with 1.5 Per Cent. Sodium Chloride Solution on Renal Function and Pathology.

A study of Experiments 7, 8, 10 and 12 of Table 2, which are representative of this group of animals, shows that following the intravenous injection of a solution of isotonic sodium chloride a fair degree of diuresis was established in the different animals. The flow of urine has varied from 6 to 12 drops per minute. The systolic blood pressure in the animals of the series varied from 105 to 126 mm. of mercury.

Following the intravenous injection of 25 c. c. per kilogram of a solution of sodium carbonate equimolecular with a 1.5 per cent. solution of sodium chloride, the reserve alkali of the blood in all of the animals was at once increased above the normal. In three of the animals the reserve alkali was increased from the normal of 8.0 to 8.2, in two animals from 8.1 to 8.2, while in the remaining animal the increase was from 8.0 to 8.15. Immediately following this degree of disturbance in the acid-base equilibrium of the blood there was established a free diuresis by all of the animals. The maximum formation of urine occurred in the animal of Experiment 7. Following the intravenous administration of an isotonic solution of sodium chloride to this animal the flow of urine was only 7 drops per minute. After the intravenous injection of an equal volume per kilogram of the sodium carbonate solution the flow of urine increased from 7 to 42 drops per minute. Associated with the free diuretic effect obtained from such a solution of sodium carbonate there occurred a rapid depletion in the alkali reserve of the blood, so that by the end of the first hour of the experiments the reserve alkali of the blood in all of the animals with two exceptions had returned to the normal. At this stage of the experiments there had occurred a moderate increase in the systolic blood pressure in all of the animals. The maximum rise in pressure of 23 mm. of mercury developed in the animal of Experiment 10.

At the end of the first hour of the experiments, while the animals were freely diuretic and in the process of establishing a normal acid-base equilibrium of the blood, a second injection of the sodium carbonate solution was given. The result obtained from the second administration of the alkali was to increase the alkali reserve of the blood again to a point beyond the normal. The increase did not exceed in any of the animals the readings obtained from the first injection.

The effect of a second injection of the carbonate solution on renal function was to induce a secondary increase in urine formation which was not in excess of the amount of urine formed by the animals from the first injection of the alkaline solution.

Within half an hour following the second carbonate injection there occurred a reduction in the alkali reserve of the blood toward the normal in all of the animals except the animal of Experiment 10. In this animal the reserve alkali was not depleted but remained unchanged at a reading of 8.2.

During the remaining two hours of the experiments the animals continued to be freely diuretic, and the reserve alkali of the blood was gradually returned to the normal reading in all of the animals except those of Experiments 7 and 10. These animals had at the commencement of the experiments normal alkali reserve determinations of 8.0. At the end of the experiments the alkali reserve readings for these animals were 8.05. Such readings are within the limits of the normal.

Urine collected from all of the animals during the course of the experiments was free from albumin and casts. The urine from the animal of Experiment 8 contained diacetic acid.

Phenolsulphonephthalein determinations showed but slight reductions in the elimination of the dye when contrasted with its output by the animals before the commencement of the experiments. The maximum reduction in the elimination occurred in the animal of Experiment 10. The normal elimination for this animal was 80 per cent. At the termination of the experiment the output of the dye in a two hour period was 68 per cent.

STUDY . TABLE 2.

Normal Dogs. The Effect of Alkaline Solutions of Differen Molecular Concentration on Renal Function and Pathology.

Number of Experiment	Urine	Phthalein 2 hour period	R.pH	Gréhant's Anesthetic 60%	Urine per minute Bl. Pres. mm. Hg	Alkaline sol. 25cc. per kg.	R.pH end of 1st half hour	Urine per minute Bl Pres. mm. Hg	R.pH end of 2nd half hour	Urine per minute Bl Pres mm. Hg	Alkaline sol. 25cc per kg.	R.pH end of 3rd half hour	Urine per minute Bl. Pres. mm Hg	R.pH end of 4th half hour	Urine per minute Bl Pres mm. Hg	R.pH end of 3rd hour	Urine per minute Bl Pres mm. Hg	R.pH end of 4th hour	Urine per minute Bl Pres mm Hg	Phthalein 2 hour period	Albumin and Casts	Diacetic acid
7	Normal	71%	8.0	25 cc. per kg. 0.9% NaCl	Urine 7 B. P. 120	Na_2CO_3 equimolecular with 1.5 % NaCl	8.15	Urine 42 B. P. 138	8.05	Urine 30 B. P. 130	Na_2CO_3 equimolecular with 1.5% NaCl	8.15	Urine 38 B. P. 141	8.1	Urine 26 B. P. 138	8.1	Urine 18 B. P. 140	8.05	Urine 21 B. P. 136	68%	0	0
8	Normal	63%	8.1	25 cc. per kg. 0.9% NaCl	Urine 12 B. P. 117	Na_2CO_3 equimolecular with 1.5% NaCl	8.2	Urine 36 B. P. 126	8.1	Urine 34 B. P. 116	Na_2CO_3 equimolecular with 1.5% NaCl	8.15	Urine 36 B. P. 124	8.1	Urine 33 B. P. 130	8.1	Urine 21 B. P. 121	8.1	Urine 12 B. P. 110	55%	0	Trace
10	Normal	80%	8.1	25 cc. per kg. 0.9% NaCl	Urine 10 B. P. 105	Na_2CO_3 equimolecular with 1.5% NaCl	8.2	Urine 31 B. P. 128	8.1	Urine 21 B. P. 120	Na_2CO_3 equimolecular with 1.5% NaCl	8.2	Urine 28 B. P. 131	8.2	Urine 24 B. P. 124	8.15	Urine 14 B. P. 112	8.05	Urine 8 B. P. 112	68%	0	0
12	Normal	68%	8.0	25 cc. per kg. 0.9% NaCl	Urine 6 B. P. 126	Na_2CO_3 equimolecular with 1.5% NaCl	8.1	Urine 41 B. P. 138	8.0	Urine 36 B. P. 130	Na_2CO_3 equimolecular with 1.5% NaCl	8.15	Urine 40 B. P. 138	8.1	Urine 30 B. P. 130	8.05	Urine 17 B. P. 121	8.0	Urine 8 B. P. 112	60%	0	0
14	Normal	70%	8.1	25 cc. per kg. 0.9% NaCl	Urine 11 B. P. 130	Na_2CO_3 equimolecular with 3% NaCl	8.3	Urine 31 B. P. 142	8.2	Urine 30 B. P. 138	Na_2CO_3 equimolecular with 3% NaCl	8.3	Urine 36 B. P. 130	8.3	Urine 12 B. P. 130	8.2	Urine 6 B. P. 128	8.2	Urine 1 B. P. 130	36%	Trace Casts	Trace
15	Normal	66%	8.1	25 cc. per kg 0.9% NaCl	Urine 14 B. P. 111	Na_2CO_3 equimolecular with 3% NaCl	8.4	Urine 38 B. P. 121	8.4	Urine 16 B. P. 120	Na_2CO_3 equimolecular with 3% NaCl	8.45	Urine 20 B. P. 122	8.4	Urine 7 B. P. 120	8.3	Urine 1 B. P. 112	8.15	Urine 0 B. P. 112	24%	Trace Casts	0
17	Normal	75%	8.1	25 cc. per kg 0.9% NaCl	Urine 6 B. P. 131	Na_2CO_3 equimolecular with 3% NaCl	8.3	Urine 34 B. P. 138	8.2	Urine 30 B. P. 128	Na_2CO_3 equimolecular with 3% NaCl	8.35	Urine 36 B. P. 132	8.2	Urine 28 B. P. 130	8.1	Urine 14 B. P. 126	8.1	Urine 7 B. P. 120	55%	Trace No Casts	Trace
18	Normal	60%	8.0	25 cc. per kg 0.9% NaCl	Urine 10 B. P. 105	Na_2CO_3 equimolecular with 3% NaCl	8.2	Urine 23 B. P. 118	8.1	Urine 20 B. P. 114	Na_2CO_3 equimolecular with 3% NaCl	8.3	Urine 24 B. P. 120	8.1	Urine 10 B. P. 114	8.1	Urine 3 B. P. 110	8.1	Urine 1 B. P. 111	30%	Trace No Casts	0
19	Normal	68%	8.1	25 cc. per kg 0.9% NaCl	Urine 7 B. P. 114	Na_2CO_3 equimolecular with 3% NaCl	8.25	Urine 31 B. P. 120	8.15	Urine 26 B. P. 118	Na_2CO_3 equimolecular with 3% NaCl	8.3	Urine 28 B. P. 126	8.2	Urine 6 B. P. 118	8.2	Urine 3 B. P. 118	8.1	Urine 0 B. P. 110	25%	Trace Casts	Trace
20	Normal	58%	8.0	25 cc. per kg. 0.9% NaCl	Urine 7 B. P. 131	Na_2CO_3 equimolecular with 3% NaCl	8.3	Urine 24 B. P. 146	8.2	Urine 18 B. P. 140	Na_2CO_3 equimolecular with 3% NaCl	8.35	Urine 20 B. P. 144	8.3	Urine 14 B. P. 129	8.2	Urine 1 B. P. 135	8.2	Urine 1 in 2 min B. P. 130	20%	Heavy precipitate No casts	0
21	Normal	74%	8.0	25 cc per kg. 0.9% NaCl	Urine 15 B. P. 118	Na_2CO_3 equimolecular with 3% NaCl	8.3	Urine 43 B. P. 129	8.1	Urine 30 B. P. 125	Na_2CO_3 equimolecular with 3% NaCl	8.4	Urine 36 B. P. 136	8.3	Urine 10 B. P. 130	8.2	Urine 10 B. P. 120	8.1	Urine 4 B. P. 107	38%	Trace Casts	0
22	Normal	60%	8.1	25 cc. per kg 0.9% NaCl	Urine 13 B. P. 124	Na_2CO_3 equimolecular with 3% NaCl	8.25	Urine 36 B. P. 136	8.1	Urine 24 B. P. 130	Na_2CO_3 equimolecular with 3% NaCl	8.2	Urine 41 B. P. 142	8.2	Urine 27 B. P. 136	8.1	Urine 16 B. P. 130	8.1	Urine 10 B. P. 118	40%	Trace No casts	Trace

The histological study of tissue obtained from the kidneys of these animals has in general shown no evidence of injury. Stainable lipoid material does not appear in the convoluted tubule cells and can rarely be demonstrated in the cells of the loops of Henle. The epithelium lining the convoluted tubules appears shrunken and stains uniformly. The nuclei are hyperchromatic. Fig. 3, Study 1.

Conclusions Concerning the Group of Normal Animals That Received a Solution of Sodium Carbonate Equimolecular with a 1.5 Per Cent. Solution of Sodium Chloride.

1. Solutions of sodium carbonate equimolecular with a 1.5 per cent. solution of sodium chloride when given intravenously to normal dogs fail to induce such a degree of disturbance in the physico-chemical state of the blood as to render the kidney unable to readjust the altered physical state. The kidney responds to this degree of change in its blood chemical environment by establishing a free diuresis, and associated with this response the reserve alkali of the blood is reduced to the normal.

2. The degree of disturbance induced in the physico-chemical state of the blood by injections of such solutions fails to express itself in terms of an injury to the kidney. Urine formed during such periods is free from both albumin and casts and the elimination of phenolsulphonephthalein shows no more reduction than is induced by an anesthesia extending over a four hour period of experimentation.

The Effect of a Solution of Sodium Carbonate Equimolecular with a 3 Per Cent. Solution of Sodium Chloride on Renal Function and Pathology in Normal Dogs.

The ten animals studied under the influence of this stronger solution of sodium carbonate were normal in so far as the functional response of the kidney was concerned. Urine from these animals was free from albumin and casts. The elimination of phenolsulphonephthalein by the respective animals varied from a maximum output of 75 per cent. to a minimum output of 58 per cent.

The reserve alkali of the blood has been normal. In the different animals the readings have varied from 8.0 to 8.1.

Before the use of the sodium carbonate solutions, the intravenous injection of an isotonic solution of sodium chloride induced a fair degree of diuresis in all of the animals. The formation of urine by the different animals varied from 6 to 15 drops per minute. Such injections caused a rise in systemic blood pressure which has varied in the different animals from 105 to 131 mm. of mercury.

Following the intravenous injections of the stronger solution of sodium carbonate, observations have been made during the four hour period of the experiments at the intervals previously indicated. The results obtained from these stronger alkaline solutions should be contrasted with the observations previously made when animals were subjected to the action of a solution of sodium carbonate equimolecular with a 1.5 per cent. solution of sodium chloride.

The immediate effect of injecting intravenously a solution of sodium carbonate equimolecular with a 3 per cent. solution of sodium chloride is to cause an abrupt and marked rise in the alkali reserve of the blood. The maximum increase in the alkali reserve was from a normal reading of 8.1 to 8.4. The minimum increase was from a normal reading of 8.1 to 8.25.

Associated with this disturbance in the physico-chemical state of the blood is the fact that all of the animals have become freely diuretic. The degree of diuresis has varied in the different animals from 23 to 43 drops per minute. The most marked diuretic effect was obtained in the animal of Experiment 17, in which a urine flow of 6 drops per minute, that was obtained from the use of 25 c. c. per kilogram of isotonic sodium chloride solution, was increased to 34 drops per minute from the use of a solution of sodium carbonate. The use of such solutions of sodium carbonate have induced a moderate rise in systolic blood pressure in all of the animals. The maximum rise of 12 mm. of mercury occurred in the animal of Experiment 14.

Associated with the profuse flow of urine provoked by such solutions there has occurred a rapid reduction in the reserve alkali of the blood in all of the animals. However, in only one of the animals was the normal acid-base equilibrium of the blood re-established in the first hour period of the experiment. This result differs from the effect obtained when a solution of sodium carbonate equimolecular with a 1.5 per cent. solution of sodium chloride was used. When the weaker solution was employed, all of the animals were able within the first hour of the experiment to reduce the reserve alkali of the blood either to the normal reading obtained for the animal before the commencement of the experiment, or to a reading which was within the limit of the normal.

At the end of the first hour of the experiment, with a freely diuretic state established in the animals, a second injection of the stronger solution of sodium carbonate was given. This injection resulted in a secondary increase in the alkali reserve of the blood which in all of the animals gave a higher reading than that obtained from the first injection. The maximum increase in the alkali reserve of 8.45 was obtained in the animal of Experiment 15.

Following this secondary disturbance in the acid-base equilibrium of the blood by a solution of sodium carbonate of the same volume and molecular concentration as the first solution, and with a rise in systemic blood pressure in all of the animals, there has developed but a slight increase in urine formation. The greatest increase of 17 drops per minute occurred in the animal of Experiment 22. In the other animals the increase in urine varied from 2 to 6 drops per minute.

From these observations it would appear that when the acid-base equilibrium of the blood was altered by the first injection of a solution of sodium carbonate equimolecular with a 3 per cent. solution of sodium chloride, so that the kidney was unable to restore this equilibrium to the normal, the persistence of such an alteration in the physico-chemical state of the blood expressed itself locally in the kidney by rendering the kidney unable to react to a second injection of such a solution with the same degree of functional response as it was shown to possess at an earlier stage in the experiments.

During the remaining two hours of the experiments there occurred a very gradual decrease in the alkali reserve of the blood. Only three of the ten animals were able during this period to re-establish their normal acid-base equilibrium. The remaining seven animals of the series at the termination of the experiments had a reserve alkali of the blood which varied from 8.1 to 8.2.

Urine formation during this period progressively decreased, so that at the end of the experiments two of the animals were anuric, one animal was forming 1 drop of urine every two minutes, and two of the animals 1 drop per minute. The largest output of urine by any of the animals at this period was 10 drops per minute.

Changes similar in character to those just recorded as developing during the last two hours of the experiments, were observed in the previously discussed group of normal dogs that received a solution of sodium carbonate equimolecular with a 1.5 per cent. solution of sodium chloride. However, in this former group of experiments none of the animals became anuric, the reduction in urine formation was not so marked, and all of the animals during the last two hours of the experiments were able to re-establish a normal acid-base equilibrium of the blood.

A study of the urine collected during the course of the experiments in which the animals received the stronger carbonate solutions shows that all of the animals developed an albuminuria and that casts occurred in the urine of four of the animals.

The elimination of phenolsulphonephthalein has been reduced in all of the experiments. The most marked reduction has occurred in the animal of Experiment 20, in which the elimination of the dye was reduced from the normal of 58 per cent. to 20 per cent. The urine from four of the animals contained diacetic acid.

The histological study of tissue from the kidneys of these animals which received the stronger solution of sodium carbonate shows in general a marked degree of cloudy swelling of the tubular epithelium. The epithelium of the convoluted tubules shows this change to an advanced degree, and in addition many of the cells are vacuolated and undergoing necrosis. Fig. 4, Study 1.

Conclusions Concerning the Group of Normal Animals That Received a Solution of Sodium Carbonate Equimolecular with a 3 Per Cent. Solution of Sodium Chloride.

1. A solution of sodium carbonate equimolecular with a 3 per cent. solution of sodium chloride when given intravenously to normal dogs effects a disturbance in the animals which differs only in degree from that obtained when a weaker solution of sodium carbonate equimolecular with a 1.5 per cent. solution of sodium chloride is employed. The stronger solutions induce a disturbance in the physico-chemical state of the blood of such a degree that the kidney is in general unable to readjust this altered physico-chemical state and establish a normal blood chemical environment for the organism.

2. The inability of the organism to effect such a readjustment is shown locally by the development of a kidney injury which expresses itself functionally in the appearance of albumin and casts in the urine, a decrease in the elimination of phenolsulphonephthalein, and by a decrease in urine formation or the establishment of an anuria.

3. These experiments in which solutions of different molecular concentrations of sodium carbonate have been employed indicate that the normal organism, very largely through the activity of the kidneys, is able to readjust to the normal a certain degree of disturbance in the acid-base equilibrium of the blood without the kidney becoming injured during such an adjustment. When, however, the physico-chemical state of the blood undergoes too great a departure from the normal from the use of such an alkaline solution, the environment of the kidney becomes so changed that its functional response is interfered with and a normal acid-base equilibrium of the blood is not established.

4. In normal dogs in which there has developed from the use of an alkaline solution or a N/2 solution of hydrochloric acid an inability to effect a readjustment in the acid-base equilibrium of the blood, the type of renal injury is in general the same. This injury is primarily one of cloudy swelling of the renal epithelium

which is most marked in the cells of the convoluted tubules. The more advanced changes of degeneration consist in edema, vacuolation and more rarely an early necrosis of the tubular epithelium.

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DESCRIPTION OF FIGURES.

STUDY I.

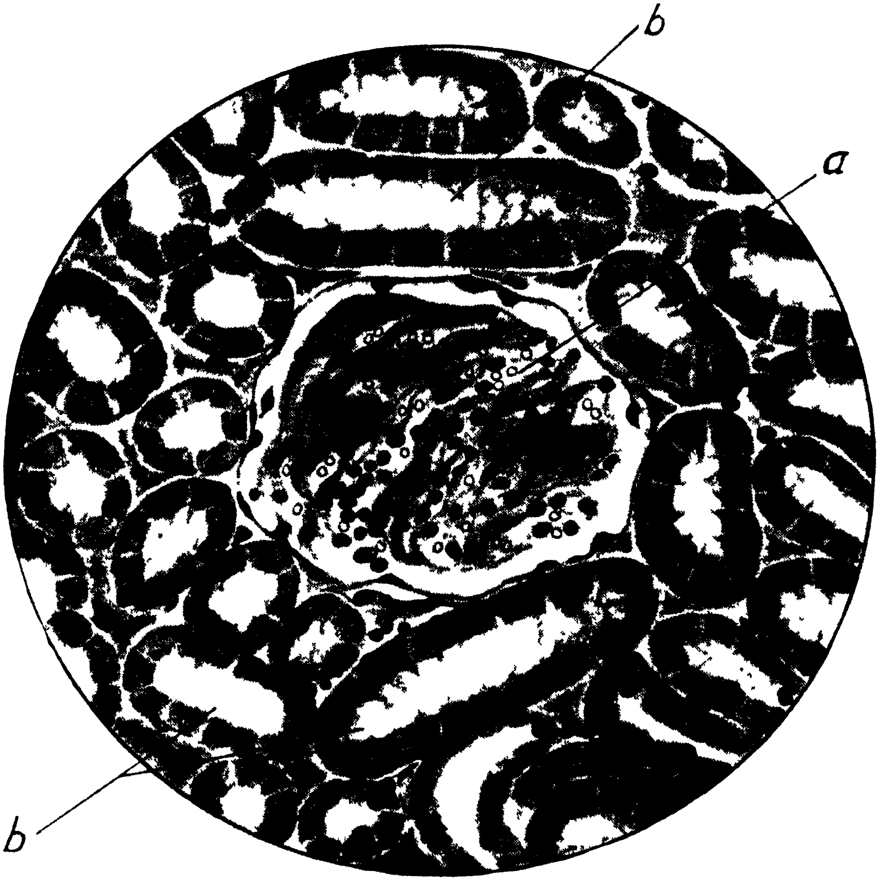

Fig. 1. Camera lucida drawing. Leitz Oc. 2, obj. 6.

The figure is from the kidney of the normal control animal of Experiment 4, Study 1, Table 1.

The animal was anesthetized by Gréhant's anesthetic for a period of four hours. With the completion of a satisfactory state of surgical anesthesia the animal was given 25 c. c. per kilogram of a 0.9 per cent solution of sodium chloride. The animal remained freely diuretic throughout the experiment. The flow of urine at the end of the experiment was 11 drops per minute. The elimination of phenolsulphonephthalein was only reduced from the normal of 65 per cent. to 50 per cent. No albumin or casts appeared in the urine. The reserve alkali of the blood was normal at the termination of the experiment. At A, is shown a normal glomerulus. At B, are shown convoluted tubules in which the epithelium appears granular but shows only slight swelling. The nuclei of the cells stain well. The epithelium shows no evidence of vacuolation or necrosis.

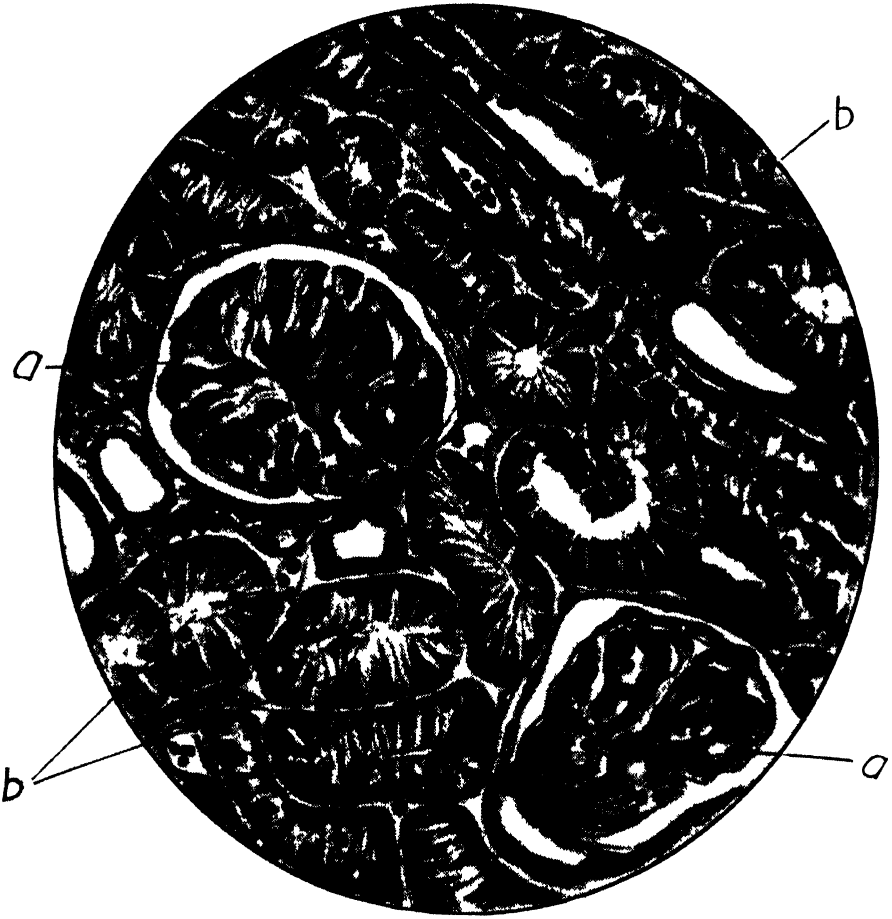

FIG 2. Camera lucida drawing Leitz Oc 2, obj. 6

The figure is from the kidney of the animal of Experiment 14, Study I, Table 1. The animal received two injections of a solution of N 2 hydrochloric acid. The animal was unable to re-establish and maintain a normal acid-base equilibrium of the blood following such injections. The reserve alkali of the blood at the termination of the experiment was 7.95. Albumin and casts appeared in the urine. The elimination of phenolsulphonephthalein was reduced from the normal output of 60 per cent. to 35 per cent. At the termination of the experiment the animal was forming only 1 drop of urine every two minutes. At A, are shown normal glomeruli. At B, are shown convoluted tubules with edematous and vacuolated epithelium. In some of the tubules the edema is so severe as to occlude the lumen of the tubules. The nuclei of the cells vary in the intensity of their staining power.

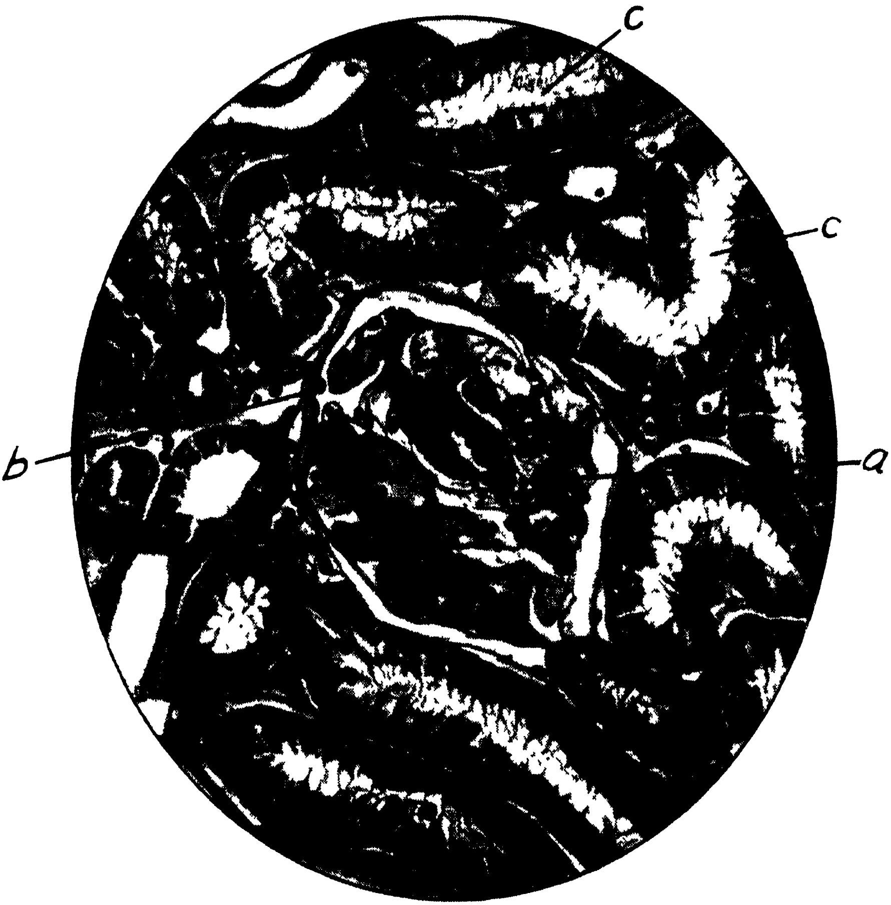

Fig. 3 Camera lucida drawing. Leitz Oc. 2, obj. 6.

The figure is from the kidney of the animal of Experiment 8, Study 1, Table 2. The animal received two intravenous injections of a solution of sodium carbonate equimolecular with a 1 5 per cent solution of sodium chloride. The animal was able to establish and maintain the normal reserve alkali of the blood of 8.1. No albumin or casts appeared in the urine. The elimination of phenolsulphonephthalein during the experiment was only reduced from the normal output of 63 per cent to 55 per cent. At the termination of the experiment the animal was forming 12 drops of urine per minute.

At A, is shown a normal glomerulus, and at B, the capsule of the glomerulus. At C, are shown convoluted tubules in a good state of preservation. The epithelium of such tubules is not swollen. The lumen of the tubules is prominent. The nuclei of the cells stain intensely.

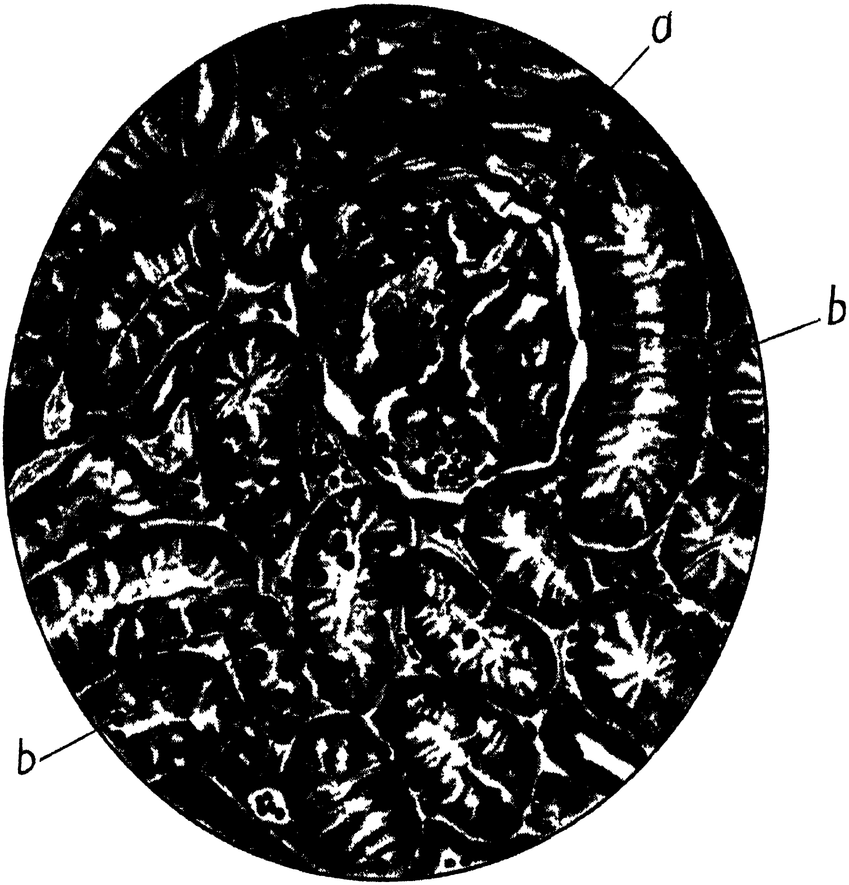

Fig 4 Camera lucida drawing. Leitz Oc 2, obj 6

The figure is from the kidney of the animal of Experiment 15, Study 1, Table 2. The animal received two intravenous injections of a solution of sodium carbonate equimolecular with a 3 per cent solution of sodium chloride The animal was unable to establish and maintain a normal acid-base equilibrium of the blood Albumin and casts appeared in the urine. The elimination of phenolsulphonephthalein was reduced from the normal of 66 per cent to 24 per cent At the termination of the experiment the animal was anuric.

At A, is shown a normal glomerulus At B, are shown convoluted tubules with the epithelium edematous and vacuolated. The lumina of many of the tubules are closed by the edematous cells. The nuclei of the cells vary in their staining power.

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STUDIES CONCERNING THE INFLUENCE OF A DISTURBANCE IN THE ACID-BASE EQUILIBRIUM OF THE BLOOD ON RENAL FUNCTION AND PATHOLOGY.*

Study II. The Effect of Acid and Alkaline Solutions on Renal Function and Pathology in Naturally Nephropathic Dogs.

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In the first study of this series an investigation was made of the stability of the acid-base equilibrium of the blood in normal dogs when anesthetized by Gréhant's anesthetic over a period of four hours and of the ability of such animals to readjust this fundamental reaction of the blood when it was disturbed by the intravenous administration of an acid or an alkaline solution. The pathological effect of such solutions was observed by a study of renal function and the histological changes induced in the kidney.

In the following study similar observations will be made on the disturbance induced in naturally nephropathic animals by the introduction of such solutions, of the ability of a previously damaged kidney to readjust a disturbance in the acid-base equilibrium of the blood, and of the changes which such solutions induce in the functional and pathological response of the kidney.

In recent years numerous investigations have appeared which have had as their object a study of the acid-base equilibrium of the blood in the various types of nephropathies and of the relationship which such a disturbance has to certain changes in renal function. These studies have been very largely concerned with the development of the acid intoxication which occurs in such renal injuries and with a discussion as to whether such a depletion in the alkali reserve of the blood should be considered as the cause for certain

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renal injuries, or whether the disturbance should be interpreted as a retention intoxication. The investigation which is to follow is not primarily concerned with these questions, but is concerned with the relative ability of the naturally nephropathic kidney as contrasted with the normal kidney to readjust such a fundamental disturbance in the blood chemical environment of the organism. The study, furthermore, takes into consideration the severity of the pathological changes developing in the kidney of such animals and the degree to which the functional response of the kidney is interfered with.

The earlier literature dealing with the question of the development of an acid intoxication in various types of nephropathic processes has been reviewed by Ewing,[1] and the more recent literature by Sellards.[2] In the present paper references will be made only to more recent papers which deal with the general question of the disturbance in the acid-base equilibrium of the blood in nephropathic animals.

In 1909 Von Hösslin[3] observed a rather definite relationship between the acidity of the urine and the amount of albumin and casts in the urine. Later than this Fischer,[4] as a result of his work on edema, applied his findings to certain changes developing in the kidney in various forms of nephritis and emphasized the importance of the acidosis occurring in such states. In 1913 Palmer and Henderson,[5] following a series of clinical studies on the acid-base equilibrium of the blood and the nature of acidosis, decided that a condition of acidosis existed in a great variey of pathological conditions. They furthermore made suggestions concerning the rational use of alkalies as therapeutic agents. At a later date the same investigators[6] demonstrated in a series of experiments the constant occurrence of an acidosis in one type of nephritis and of its frequent occurrence in other types. In these studies they were able to demonstrate a retention of alkali following the use of sodium bicarbonate. In 1915 Peabody[7] made a study of the acidosis of chronic nephritis in connection with functional renal tests and came to the conclusion that the acidosis in such conditions was a retention phenomenon. Very recently Chase and Myers[8] in a study of the acidosis of nephritis have come to the conclusion that all fatal cases of nephritis with marked nitrogen retention have a severe acidosis, sufficient in many instances to be the cause of death.

In recent years several investigations have been made in this laboratory concerning the stability of the acid-base equilibrium of the blood in animals of different age periods,[9] and in naturally nephropathic animals. These observations have been extended to a study of the susceptibility of such animals to the toxic effect of the general anesthetics[10,11] and the degree of disturbance induced in the acid-base equilibrium of the blood by the use of such anesthetic substances. Other studies have dealt with the restoration of the acid-base equilibrium of the blood in animals recovering from an acute nephropathic process[12,13] and of the disturbance which is induced in this equilibrium when an acute injury is superimposed on a chronic renal injury.[14]

In 1903 Pearce,[15] in an experimental study of nephrotoxins, used in his investigation the sera from dogs with a spontaneous nephritis. Later, in 1908, Ophüls[16] noticed that chronic nephritis in dogs is a common disease. Dayton,[17] in an investigation of the frequency of such a condition in dogs, found in a study of twenty-one animals only one dog with what he considered normal kidneys. In 1916 an extensive study was made in this laboratory of the naturally acquired nephropathy of the dog and of the physiological response of such kidneys to diuretic substances.[18,19] In these studies the frequency of the occcurrence of a chronic nephropathy in the dog was confirmed and the various nephropathic processes were classified for purposes of study. The classification showed very clearly that with few exceptions the chronic kidney injury in the dog is primarily a glomerulonephropathy and that extensive changes may develop in the glomeruli before the tubules become implicated in the pathological process. The animals employed in the following study have had this type of chronic naturally-acquired kidney injury.

The Effect of a N/2 Solution of Hydrochloric Acid on Renal Function and Pathology in Naturally Nephropathic Dogs.

Fifteen naturally nephropathic animals were used in this series of experiments. The preliminary observations and the experimental procedures are identical with those outlined in Study 1, in which normal dogs were employed. Four of these animals were used for control experiments. They were anesthetized by Gréhant's anesthetic and given intravenously 25 c. c. per kilogram of a 0.9 per cent. solution of sodium chloride. The remaining animals were anesthetized and given a similar solution of sodium chloride and at two periods of the experiments were given intravenously 5 c.c. per kilogram of a N/2 solution of hydrochloric acid. The observations on all of the control animals and on eight of the animals subjected to the action of a solution of hydrochloric acid have been included in Table 1 of the present study.

The observations made on these animals during the period of preliminary study show them to be naturally nephropathic. The urine from all of the animals contained albumin and with one exception tube casts. The appearance of the albumin was variable. In three of the fifteen animals its presence was not constant, but varied from day to day.

The elimination of phenolsulphonephthalein in a two hour period was uniformly below the normal and varied from a minimum output of 33 per cent. to a maximum output of 58 per cent. The reserve alkali of the blood in these naturally nephropathic animals in which the chronic pathology is largely localized in the glomeruli was normal with two exceptions. In these two animals, Experiments 7 and 12, the reserve alkali was 7.95. In such animals, with a reduction in the reserve alkali of the blood, the elimination of phenolsulphonephthalein was low, and the amount of albumin in the urine or the number of casts was more marked than in any of the other animals.

The animal of Experiment 7 had a reserve alkali of 7.95, and elimination of phenolsulphonephthalein of 48 per cent.; and while only a trace of albumin was present in the urine, there were very numerous hyaline and finely granular casts. The animal of Experiment 12 had a reserve alkali of 7.95, and elimination of phenolsulphonephthalein of 33 per cent., and a heavy trace of albumin with casts.

Control Experiments with Naturally Nephropathic Animals.

These animals were not subjected to the action of a solution of hydrochloric acid. Following the establishment of a state of anesthesia from Gréhant's anesthetic and after the administration of the usual solution of isotonic sodium chloride, a fair degree of diuresis was obtained in the various animals. The flow of urine varied from 10 to 21 drops per minute. The systolic blood pressure in the respective animals has varied from 100 mm. to 134 mm. of mercury.

At this early period of the experiments the reserve alkali of the blood remained unchanged except in the animal of Experiment 2, in which the reserve alkali was reduced from the normal reading of 8.05 to 8.0.

By the end of the second half hour period of the experiments very little change had taken place in the rate of urine formation or in the systolic blood pressure of the different animals. The reserve alkali of the blood at this period was reduced in all of the animals except one. The maximum reduction was obtained in the animal of Experiment 1, in which the reserve alkali was reduced from the normal of 8.0 to 7.9.

At the termination of the third half hour period of the experiments, urine formation had undergone a marked reduction. The animal of Experiment 2 was anuric. Such changes in urine formation have not been associated with any marked fall in systolic blood pressure. In the anuric animal of Experiment 2, systolic blood pressure at this period of the experiment was 128 mm. of mercury, 4 mm. in excess of the normal blood pressure reading for the animal.

In the animal of Experiment 5, urine formation had been reduced from the normal flow of 18 drops per minute to 11 drops per minute. The normal blood pressure for this animal was 134 mm. of mercury. Associated with the reduction in urine formation the blood pressure had fallen only to 130 mm. of mercury.

A study of the acid-base equilibrium of the blood at this period of the experiments, when urine formation is either being reduced or the animals rendered anuric, shows a marked depletion in the alkali reserve in all of the animals. The reserve alkali of the blood in the animal of Experiment 5, in which urine formation was reduced from 18 to 11 drops per minute, was reduced from 8.1 to 7.9. In the anuric animal of Experiment 2, the reserve alkali had undergone a depletion from 8.0 to 7.85.

Throughout the remainder of the experiments which lasted for four hours, there was a progressive decrease in urine formation by all of the animals. At the termination of the experiments the maximum urine formation by the animal of Experiment 5 was 4 drops per minute. The animals of Experiments 1 and 4 were forming 2 drops of urine per minute, while the animal of Experiment 2, that became anuric early in the experiment, remained anuric. The systolic blood pressure in these animals did not undergo any marked reduction the blood pressure for the different animals varied from 98 to 120 mm. of mercury.

The reserve alkali of the blood, although depleted from the normal in all of the animals, remained very constant, with one exception, after the initial depletion which occurred at the end of the third half hour period of the experiments. The readings for all of the animals, except the anuric animal of Experiment 2, were 7.9. In this latter animal the reserve alkali of the blood at the termination of the experiment was reduced to 7.8.

A study of the urine formed by the different animals during the course of the experiments showed an increase in the amount of albumin over that normally present in the urine. Diacetic acid appeared in the urine of all of the animals examined for this substance.

The elimination of phenolsulphonephthalein was reduced in all of the animals during the period of anesthesia. In the animal of Experiment 4, the elimination of the dye was only reduced to 40 per cent. from the normal output of 50 per cent. In the animal of Experiment 2, that became anuric during the period of anesthesia, the elimination of the dye was reduced from 45 per cent. to a trifle less than 10 per cent.

A study of the histological changes induced in the naturally nephropathic kidney by an anesthesia of four hours duration from Gréhant's anesthetic shows that the vascular tissue fails to develop an acute injury. The glomerular capillaries were well filled with blood when the chronic fibrous changes and hyalinization did not exclude the circulation. No exudate or actual hemorrhage was observed in the subcapsular spaces. The chronic pathology of the glomeruli consisted of those capsular and intracapillary changes common to an early or moderately advanced glomerulonephropathy.

The toxicity of the anesthetic for the kidney is expressed by changes in the tubular epithelium. The amount of stainable lipoid material is increased in the cells of the loops of Henle and in the convoluted tubule epithelium. The epithelium of the tubules in general shows cloudy swelling and a moderate grade of vacuolation. In the anuric animal of Experiment 2, the epithelium of the convoluted tubules showed an advanced grade of swelling, vacuolation, and in many of the cells a well advanced necrosis. Fig. 1. Study II.

Conclusions Concerning the Control Group of Naturally Nephropathic Animals.

1. Following an anesthesia of four hours duration from Gréhant's anesthetic in naturally nephropathic dogs, there is more evidence

of its toxic effect than develops from a similar period of anesthesia in normal dogs.

2. This increased toxic effect is shown by the naturally nephropathic animals by the anesthetic inducing an earlier and more marked disturbance in the acid-base equilibrium of the blood which is associated with a reduction in urine formation and which is not accompanied by a reduction in systolic blood pressure.

3. In normal dogs, following the initial disturbance in the acid-base equilibrium of the blood induced by the anesthetic, there occurred a readjustment of this equilibrium which was associated with an increase in urine formation.

In naturally nephropathic animals after the anesthetic has induced a disturbance in the acid-base equilibrium of the blood, there is no attempt on the part of the animals to re-establish this balance. The degree of depletion in the alkali reserve of the blood remains unchanged, or it undergoes a further reduction as the anesthesia progresses.

4. In naturally nephropathic animals in which the blood has undergone such a physico-chemical change that the kidney is no longer furnished a normal environment in which to functionate, there occurs a reduction in urine formation or the establishment of an anuria. The elimination of phenolsulphonephthalein is reduced and an increased amount of albumin appears in the urine. This altered environment of the kidney is shown histologically by the development of acute changes of a degenerative character in the tubular epithelium and by the lack of such changes in the glomeruli which are the seat of the primary chronic injury.

The Effect of a N/2 Solution of Hydrochloric Acid on Renal Function and Pathology in Naturally Nephropathic Dogs.

Eleven of the naturally nephropathic animals of this series were subjected to the same experimental technique as has been outlined for the control group of animals, and in addition at two periods of the experiments were given intravenously 5 c. c. per kilogram of a N/2 solution of hydrochloric acid. The results obtained in eight of these animals are included in Table 1, Study II.

At the completion of the anesthesia from Gréhant's anesthetic and following the intravenous injection of 25 c. c. per kilogram of a 0.9 per cent. solution of sodium chloride, these animals showed a fair degree of diuresis. The flow of urine varied in the different animals from an output of 4 to 18 drops per minute. The systemic blood pressure in the respective animals varied from a minimum pressure of 100 mm. of mercury to a maximum pressure of 140 mm. of mercury.

At this stage of the experiments the animals were given the first intravenous injection of a solution of hydrochloric acid. The effect from such an injection was to induce a marked disturbance in the acid-base equilibrium of the blood in all of the animals. The degree of depletion in the alkali reserve varied in the different animals. In the animal of Experiment 8, the reserve alkali was reduced from 8.1 to 8.0; in the animal of Experiment 10, from the normal of 8.0 to 7.8. Associated with such changes in the alkali reserve of the blood in naturally nephropathic animals, there occurred a reduction in urine formation in all of the animals with two exceptions. In two of the animals the output of urine increased 2 drops per minute. This observation is in striking contrast with the results obtained when a similar solution of hydrochloric acid was administered to normal dogs. In such animals a profuse flow of urine followed the acid injection. (Study I.)

In the naturally nephropathic animals no marked change developed in the systolic blood pressure from the first injection of the acid solution. The blood pressure readings for the respective animals varied from a minimum reading of 105 mm. of mercury in the animal of Experiment 6 to the maximum reading of 142 mm. of mercury in the animal of Experiment 14.

At the end of the second half hour period of the experiments the reserve alkali of the blood remained reduced below the normal in all of the animals. Four of the animals, as the experiments had progressed during this period, showed a progressive reduction in the reserve alkali of the blood, which was most marked in the animal of Experiment 12, in which the reserve alkali determination now gave a reading of 7.7. In the remaining animals the reserve alkali remained unchanged. In none of the animals was there any evidence of an attempt at a restoration of the acid-base equilibrium of the blood. At this period of the experiments, the end of the second half hour, two of the animals had become anuric, and with the exception of the animal of Experiment 10, in which the formation of urine remained at 2 drops per minute urine formation by all of the remaining animals had been greatly reduced.

The reduction in urine formation shows no correlation with a fall in systemic blood pressure. In the anuric animal of Experiment 12, the blood pressure at this period was 130 mm. of mercury as opposed to a pressure of 128 mm. of mercury when the animal was forming 4 drops of urine per minute.

Following these observations, at the end of the first hour of the experiments, the animals were given the second and final injection of 5 c. c. per kilogram of a N/2 solution of hydrochloric acid. The injection of the acid induced a secondary and more marked disturbance in the acid-base equilibrium of the blood than was obtained from the first injection. The maximum reduction of the alkali reserve of the blood to 7.6 occurred in the animals of Experiments 12 and 15. The minimum reduction to 7.9 developed in the animal of Experiment 11. Following this

STUDY II. TABLE 1.

Naturally Nephropathic Dogs. The Effect of a N/2 Solution of Hydrochloric Acid on Renal Function and Pathology.

Number of Experiment	Urine	Phthalein 2 hour period	R.pH	Gréhant's anesthetic 60%	Urine per minute Bl. Pres. mm. Hg	Acid or Alkali	R.pH end of 1st half hour	Urine per minute Bl. Pres. mm. Hg	R.pH end of 2nd half hour	Urine per minute Bl. Pres. mm. Hg	Acid or alkali	R.pH end of 3rd half hour	Urine per minute Bl. Pres. mm. Hg	R.pH end of 4th half hour	Urine per minute Bl. Pres. mm. Hg	R.pH end of 3rd hour	Urine per minute Bl. Pres. mm. Hg	R.pH end of 4th hour	Urine per minute Bl. Pres. mm. Hg	Phthalein 2 hour period	Albumin and Casts	Diacetic acid
1 Control	Trace of albumin Casts	56%	8.0	25 cc. per kg. 0.9% NaCl	Urine 10 B. P. 100	0	8.0	Urine 18 B. P. 114	7.9	Urine 14 B. P. 100	0	7.9	Urine 8 B. P. 102	7.9	Urine 2 B. P. 100	7.9	Urine 2 B. P. 98	7.9	Urine 2 B. P. 100	35%	Albumin increased Casts	Present
2 Control	Trace of albumin No casts	45%	8.05	25 cc. per kg. 0 9% NaCl	Urine 21 B. P. 124	0	8.0	Urine 20 B. P. 120	8.0	Urine 11 B. P. 120	0	7.85	Urine 0 B. P. 128	7 85	Urine 0 B. P. 120	7.8	Urine 0 B. P. 114	7.8	Urine 0 B. P. 110	Trace	Not examined	Not examined
4 Control	Trace of albumin Casts	50%	8.05	25 cc. per kg. 0.9% NaCl	Urine 14 B. P. 110	0	8.05	Urine 10 B. P. 113	8.05	Urine 7 B. P. 116	0	7.9	Urine 7 B. P. 112	7.9	Urine 7 B. P. 110	7.9	Urine 2 B. P. 100	7.9	Urine 2 B. P. 100	40%	Albumin increased Casts	Present
5 Control	Trace of albumin Casts	48%	8.1	25 cc. per kg. 0.9% NaCl	Urine 18 B. P. 134	0	8.1	Urine 18 B. P. 150	8.0	Urine 10 B. P. 130	0	8.0	Urine 11 B. P. 130	7 9	Urine 4 B. P. 128	7.9	Urine 4 B. P. 120	7.9	Urine 0 B. P. 120	30%	Albumin increased Casts	Present
6	Trace of albumin Casts	44%	8.0	25 cc. per kg. 0 9% NaCl	Urine 10 B. P. 100	5 cc. per kg. N/2 HCl	7.9	Urine 3 B. P. 105	7.85	Urine 3 B. P. 105	5 cc. per kg. N/2 HCl	7.75	Urine 1 B. P. 100	7.65	Urine 1 in two min B. P. 98	7.6	Urine 0 B. P. 100	7.6	Urine 0 B. P. 96	10%	Not examined	Not examined
7	Trace of albumin Casts numerous	48%	7.95	25 cc. per kg. 0.9% NaCl	Urine 12 B. P. 114	5 cc. per kg. N/2 HCl	7.85	Urine 4 B. P. 120	7.85	Urine 0 B. P. 110	5 cc. per kg. N/2 HCl	7.7	Urine 0 B. P. 110	7 6	Urine 0 B. P. 100	7.6	Urine 0 B. P. 100	7 6	Urine 0 B. P. 100	Not made	Heavy precipitate Casts	Present
8	Trace of albumin Casts	58%	8.1	25 cc per kg. 0 9% NaCl	Urine 16 B. P. 132	5 cc. per kg. N/2 HCl	8 0	Urine 16 B. P. 130	7.9	Urine 10 B. P. 120	5 cc. per kg. N/2 HCl	7.8	Urine 10 B. P. 125	7 8	Urine 8 B. P. 118	7.75	Urine 4 B P. 110	7.7	Urine 1 B. P. 111	18%	Heavy precipitate Casts	Present
10	Trace of albumin Casts	38%	8 0	25 cc. per kg. 0.9% NaCl	Urine 5 B. P. 128	5 cc. per kg. N/2 HCl	7 8	Urine 2 B. P. 130	7.8	Urine 2 B. P. 120	5 cc. per kg. N/2 HCl	7 7	Urine 2 B. P. 125	7 7	Urine 0 B. P. 118	7.7	Urine 0 B. P. 110	7.65	Urine 0 B. P. 110	Trace	Not examined	Not examined
11	Trace of albumin	45%	8.1	25 cc. per kg. 0.9% NaCl	Urine 18 B. P. 124	5 cc. per kg. N/2 HCl	8.0	Urine 20 B. P. 130	8.0	Urine 10 B. P. 120	5 cc. per kg. N/2 HCl	7.9	Urine 8 B. P. 122	7.8	Urine 5 B. P. 118	7 8	Urine 1 B. P. 114	7.8	Urine 0 B. P. 100	Trace	Heavy precipitate Casts	Present
12	Heavy trace of albumin Casts	33%	7.95	25 cc. per kg. 0.9% NaCl	Urine 4 B. P. 130	5 cc. per kg. N/2 HCl	7.8	Urine 4 B. P. 128	7.7	Urine 0 B. P. 130	5 cc. per kg. N/2 HCl	7.6	Urine 0 B. P. 120	7.6	Urine 0 B. P. 118	7 6	Urine 0 B. P. 115	7 6	Urine 0 B. P. 105	Not made	Solid mass of albumin Casts	Not examined
14	Trace of albumin No casts	42%	8.0	25 cc. per kg. 0 9% NaCl	Urine 12 B. P. 140	5 cc. per kg. N/2 HCl	7.9	Urine 14 B. P. 142	7.9	Urine 11 B. P. 133	5 cc. per kg. N/2 HCl	7.8	Urine 5 B. P. 135	7.75	Urine 1 B. P. 124	7.7	Urine 0 B. P. 120	7.7	Urine 0 B. P. 110	Trace	Not examined	Not examined
15	Trace of albumin Casts	46%	8.0	25 cc. per kg. 0.9% NaCl	Urine 8 B. P. 116	5 cc. per kg. N/2 HCl	7.85	Urine 8 B. P. 118	7.8	Urine 2 B. P. 110	5 cc. per kg. N/2 HCl	7.6	Urine 2 B. P. 110	7.6	Urine 0 B. P. 110	7 6	Urine 0 B. P. 100	7.6	Urine 0 B. P. 100	Trace	Heavy precipitate Casts	Present

degree of change in the physico-chemical state of the blood, urine formation which had previously been reduced, remained unchanged in three of the animals, Experiments 8, 10 and 15; two of the animals continued anuric, while in the remaining animals there was a further reduction in urine formation.

The blood pressure determinations in the respective animals varied from a minimum pressure of 100 mm. of mercury in the animal of Experiment 6, to a maximum blood pressure of 135 mm. of mercury in the animal of Experiment 14.

From this stage of the experiments until their termination at the end of a four hour period, the disturbance induced in the acid-base equilibrium of the blood showed either a progressive increase of the degree of disturbance or remained unchanged from that induced by the second injection of the hydrochloric acid solution. At the end of the experiments the lowest reserve alkali readings which occurred in four of the animals, Experiments 6, 7, 12 and 15, were 7.6. The highest reading of 7.8 was obtained in the animal of Experiment 11. The reserve alkali determinations for the remaining animals was found between these two extremes. There was no attempt on the part of any of the animals to re-establish a normal acid-base equilibrium of the blood.

Associated with this degree of departure from the normal in the acid-base equilibrium of the blood and with an inability to attempt a re-establishment of the equilibrium, all of the animals after the first hour of the experiments showed a marked reduction in urine formation, so that by the end of the third hour of the experiments six of the eleven animals were anuric, and by the termination of the experiments, one hour later, all of the animals, with one exception, were anuric. This animal, Experiment 8, was forming 1 drop of urine per minute.

This reduction in urine formation cannot be ascribed to an excessively low systolic blood pressure in any of the animals. At the conclusion of the experiments the systolic blood pressure in the different animals varied from a minimum of 96 mm. to a maximum of 111 mm. of mercury.

Urine collected from the animals during the course of the experiments showed a heavy precipitate of albumin and the presence of numerous hyalin and finely granular casts. The amount of albumin was in excess of that found in the urine prior to the experiments. Diacetic acid was present in the urine of all of the animals examined for this substance. In those animals in which urine formation was sufficient to permit a phenolsulphonephthalein determination, the elimination of the dye in a two hour period varied from a mere trace to a maximum output of 18 per cent.

The histological study of the kidneys of the naturally nephropathic animals anesthetized by Gréhant's anesthetic and subjected to a disturbance in the acid-base equilibrium of the blood from two injections of 5 c. c. per kilogram of a N/2 solution of hydrochloric acid showed the following changes.

The changes are similar to those described for the control group of naturally nephropathic animals which were anesthetized by Gréhant's anesthetic and which were not given the acid injections, except that the use of the acid solution in this latter group of animals very greatly increased the severity of these changes.

The glomeruli showed no evidence of an acute injury. The chronic glomerular pathology consisted of the same type of chronic changes previously described.

The tubular epithelium shows a marked increase in stainable lipoid material over that which could be demonstrated in the control group of animals.

The tubular epithelium in general has shown advanced cloudy swelling, vacuolation and an early necrosis. These changes are least marked in the epithelium of the collecting tubules and most marked in the convoluted tubule epithelium. In the latter location the epithelium is frequently so edematous as to obliterate the lumen of the tubules.

The nuclei show fragmentation and fail to stain and in such cells necrosis may be seen in an advnaced stage. Fig. 2. Study II.

Conclusions Concerning the Effect of Injections of a N/2 Solution of Hydrochloric Acid on Renal Function and Pathology in Naturally Nephropathic Dogs.

1. Naturally nephropathic animals show an early and marked susceptibility to the toxic effect of intravenous injections of N/2 solutions of hydrochloric acid. The toxic effect is first shown by a severe disturbance in the acid-base equilibrium of the blood, which is soon followed by a reduction in urine formation.

2. The response of naturally nephropathic animals to such solutions differs in several particulars from the response of normal animals. Study 1. In the first place the degree of disturbance in the acid-base equilibrium of the blood is more marked in naturally nephropathic animals than in normal animals, even though the naturally nephropathic animal may have been able to maintain prior to the experiment a normal acid-base equilibrium of the blood. In the second place, when normal animals are given such solutions of hydrochloric acid, there occurs very rapidly an attempt to reestablish the normal physico-chemical state of the blood. The increased hydrogen ion content of the blood induces a free diuresis, and, associated with the rapid output of urine, the acid-base equilibrium of the blood is either restored to the normal or an attempt is made on the part of the organism in this direction.

When such acid solutions are administered to naturally nephropathic animals with a normal acid-base equilibrium of the blood, or with a blood slightly depleted in its alkali reserve, the disturbance induced in the acid-base equilibrium is so far reaching in its effect, the environment of the kidneys in terms of the physico-chemical state of their blood supply is so changed, that either no diuretic effect is obtained or the flow of urine is but slightly increased for a short period.

It would appear that in naturally nephropathic animals, such as have been used in this study, some mechanism in the kidney is normally under the strain of in part maintaining a normal acid-base equilibrium of the blood for the organism as a whole, and for the functional response of the kidney in particular. When this mechanism is subjected to the additional strain induced by a period of anesthesia, plus the introduction of an acid solution, it becomes ineffective, and this lack in its effectiveness is shown by a more marked reduction in the alkali reserve of the blood than occurs in normal animals. There is an inability on the part of the animal to readjust the physico-chemical disturbance in the blood in so far as a restoration of the acid-base equilibrium of the blood is concerned.

3. A further study of the course of the experiments in naturally nephropathic animals shows that after the acid-base equilibrium of the blood has once undergone the degree of disturbance induced by the first injection of an acid solution, and the blood chemical environment of the kidney has become so changed that the renal function in this environment is either reduced or arrested, the animals are throughout the experiments unable to readjust a balance between the hydrogen and hydroxyl ion content of the blood. As the experiments progress, the disturbance in the acid-base equilibrium of the blood increases, and associated with this further alteration in the physico-chemical state of the blood the kidneys cease to act as functional units. With two exceptions, all of the animals of the series were anuric at the termination of the experiments.

4. The greater toxicity of an acid solution for the naturally nephropathic kidney than for the normal kidney is furthermore shown anatomically by the more extensive degenerative changes in the tubular epithelium and functionally by the relative inability of such animals to eliminate phenolsulphonephthalein.

The Effect of Alkaline Solutions of Different Molecular Concentration on Renal Function and Pathology in Naturally Nephropathic Dogs.

Eighteen naturally nephropathic animals were used in this series of experiments. Eight of these animals were studied following the intravenous injection of 25 c. c. per kilogram of a solution of sodium carbonate equimolecular with a 1.5 per cent. solution of sodium chloride. The remaining animals of the series received 25 c. c. per kilogram of a solution of sodium carbonate equimolecular with a 3 per cent. solution of sodium carbonate. The effect of these solutions was observed over the usual experimental period of four hours. The results obtained in twelve representative experiments are included in Table 2. Study II.

The Effect of a Solution of Sodium Carbonate Equimolecular with a 1.5 Per Cent. Solution of Sodium Chloride on Renal Function and Pathology.

Of the eight animals studied under the influence of a solution of sodium carbonate of the above-mentioned strength, all were found to be naturally nephropathic. The urine contained albumin and casts. The elimination of phenolsulphonephthalein in a two hour period varied in the respective animals from 38 to 50 per cent. The reserve alkali of the blood in the animal of Experiment 5 was 7.95. In the remaining animals these readings were within the normal and varied from 8.0 to 8.05.

Following the development of a state of anesthesia, the animals were given the usual preliminary injection of 25 c. c. per kilogram of isotonic sodium chloride solution. There developed a fair degree of diuresis which varied in the different animals from an output of 6 drops to 17 drops of urine per minute. The systolic blood pressure varied from 100 mm. of mercury to 132 mm. of mercury.

At this period of the experiments the animals were given intravenously 25 c. c. per kilogram of a solution of sodium carbonate equimolecular with 1.5 per cent. solution of sodium chloride. The immediate effect of such injections was to increase the alkali reserve of the blood and to induce a very free flow of urine. The alkali reserve determinations made half an hour after such injections varied from 8.15 to 8.2. The maximum increase in the alkali reserve of the blood which occurred in the animals of Experiments 5 and 6 was from the normal readings of 7.95 and 8.0 to 8.15 and 8.2.

The urine flow in the different animals varied from 24 to 34 drops per minute. The most marked increase occurred in the animal of Experiment 6, in which the normal urine flow was increased from 10 to 31 drops per minute. The systolic blood pressure was raised in all of the animals by the injection of the alkaline solution. The blood pressure in the different animals varied from 118 to 142 mm. of mercury.

By the end of the second half hour of the experiments the reserve alkali of the blood had undergone a marked depletion in all of the animals, and in the animal of Experiment 6 the normal acid-base equilibrium of the blood had been re-established. The animals continued to remain freely diuretic during this early period of the experiments. There occurred but slight reductions in the systolic blood pressure.

At this stage of the experiments, the end of the second half hour period, the injection of the alkaline solution was repeated. As was the case from the initial injection of such a solution, the acid-base equilibrium of the blood was disturbed by the increase in its hydroxyl ion content. The maximum increase in the reserve alkali occurred in the animals of Experiments 4 and 6, in which determinations of 8.25 were obtained. The systolic blood pressure in all of the animals was increased.

The results so far obtained from the second injection of a solution of sodium carbonate are similar in character to the effect from the first carbonate injection. The influence, however, which the second injection has on urine formation differs from the previously recorded results. The flow of urine was increased in only one animal of the series. In this animal, Experiment 5, there existed before the beginning of the experiment a reduction in the reserve alkali of the blood. The second carbonate injection in this animal raised the alkali reserve of the blood only to 8.15, and the urine formation increased from 10 to 21 drops per minute. In the remaining animals in which the reserve alkali was increased to, or beyond 8.2, urine formation was either not increased or a reduction in urine formation occurred.

Commencing with the fourth half hour period of the experiments, the results obtained for the remaining two hours have been of the same character for all of the animals.

There is a gradual attempt at a restoration of the normal acid-base equilibrium of the blood. This has not been accomplished by any of these naturally nephropathic animals. The alkali reserve readings at the termination of the experiments varied from 8.0 to 8.2. The reading of 8.0 was obtained in the animal of Experiment 5, in which before the beginning of the experiment the alkali reserve was depleted to 7.95.

Associated with this persisting disturbance in the physico-chemical state of the blood, even though the systemic blood pressure of the animals was well maintained until the end of the experiments, there was a gradual reduction in urine formation. None of the animals became anuric.

Urine formation at the termination of the experiments varied from 1 to 5 drops per minute. Urine collected during the experiments showed a trace of albumin but no casts. The amount of albumin was apparently not increased during the period of experimentation. In the animal of Experiment 5, in which the reserve alkali was 7.95 prior to the experiment and 8.0 at its termination, the amount of albumin in the urine was reduced. The urine from two of the animals showed a trace of diacetic acid.

The elimination of phenolsulphonephthalein was so lightly reduced in all of the animals. This reduction, with one exception, was not in excess of that obtained from a period of anesthesia by Gréhant's anesthetic in naturally nephropathic animals that had not received an alkaline solution. (Table 1. Study II.)

The elimination of the dye varied from a minimum output of 20 per cent. to a maximum output of 40 per cent. The most marked reduction occurred in the animal of Experiment 4, in which the acid-base equilibrium of the blood at the termination of the experiment showed the most marked degree of disturbance. The reserve alkali of the blood at this time was 8.2, whereas the normal reading for this animal was 8.05. The normal elimination of phenolsulphonephthalein by this animal was 50 per cent. At the end of the experiment the output was 20 per cent.

The histological study of the kidneys from these naturally nephropathic animals shows the same type of chronic glomerular pathology that has been previously described. As has been noted in a previous publication,[20] when such animals are given an alkaline solution, the amount of stainable lipoid material which can be demonstrated in the renal epithelium is very greatly reduced. The principle changes developing in the kidney take place in the tubular epithelium and mainly in the convoluted tubule cells. In such cells there has developed a moderate grade of cloudy swelling with the nuclei of the cells staining imperfectly. Vacuolation has been occasionally noted. The cells have not shown evidence of necrosis. Fig. 3. Study II.

The most marked changes of this character developed in the kidneys of the animal of Experiment 4, in which there persisted the most marked disturbance in the acid-base equilibrium of the blood and in which the elimination of phenolsulphonephthalein was reduced to 20 per cent. in a two hour period.

Conclusions Concerning the Effect of a Solution of Sodium Carbonate Equimolecular with a 1.5 Per Cent. Solution of Sodium Chloride on Renal Function and Pathology in Naturally Nephropathic Dogs.

1. When naturally nephropathic animals are given such a solution of sodium carbonate there occurs a disturbance in the acid-base equilibrium of the blood from the introduction of an excess of hydroxyl ions. Following the first injection of such a solution in these animals, in which the chronic kidney injury is largely confined to the glomeruli, the kidney responds to this degree of departure from the normal in the physico-chemical state of its blood supply by a profuse diuresis in an attempt to restore a normal acid-base equilibrium. This restoration has been accomplished in two of the eight animals employed in the experiments, and in all of the animals the normal alkali reserve of the blood was reduced to very near the normal reading for the respective animal.

STUDY II. TABLE 2.

Naturally Nephropathic Dogs. The Effect of Alkaline Solutions of Different Molecular Concentrations on Renal Function and Pathology.

Number of experiment	Urine	Phthalein 2 hour period	R.pH	Gréhant's anesthetic 60%	Urine per minute Bl Pres. mm. Hg	Alkaline solution 25 cc. per kg.	R.pH end of 1st half hour	Urine per minute Bl. Pres. mm. Hg	R.pH end of 2nd half hour	Urine per minute Bl. Pres. mm. Hg	Alkaline solution 25 cc. per kg.	R.pH end of 3rd half hour	Urine per minute Bl. Pres. mm. Hg	R.pH end of 4th half hour	Urine per minute Bl. Pres. mm. Hg	R.pH end of 3rd hour	Urine per minute Bl. Pres. mm. Hg	R.pH end of 4th hour	Urine per minute Bl. Pres. mm. Hg	Phthalein 2 hour period	Albumin and Casts	Diacetic acid
2	Trace of albumin Casts	42%	8.0	25 cc. per kg. 0.9% NaCl	Urine 14 B. P. 100	Na_2CO_3 equimol. with 1.5% NaCl	8.15	Urine 24 B. P. 118	8.05	Urine 20 B. P. 118	Na_2CO_3 equimol. with 1.5% NaCl	8.2	Urine 20 B. P. 124	8.1	Urine 15 B. P. 114	8.1	Urine 11 B. P. 115	8.1	Urine 5 B. P. 110	38%	Trace No casts	Trace
4	Trace of albumin No casts	50%	8.05	25 cc per kg. 0.9% NaCl	Urine 17 B. P. 131	Na_2CO_3 equimol. with 1.5% NaCl	8.2	Urine 34 B. P. 142	8.1	Urine 12 B. P. 130	Na_2CO_3 equimol. with 1.5% NaCl	8.25	Urine 10 B. P. 140	8.2	Urine 6 B. P. 130	8.2	Urine 1 B. P. 120	8.2	Urine 1 B. P. 118	20%	Trace No casts	0
5	Heavy trace albumin Casts	38%	7.95	25 cc. per kg. 0.9% NaCl	Urine 6 B. P. 114	Na_2CO_3 equimol. with 1.5% NaCl	8.15	Urine 26 B. P. 127	8.0	Urine 10 B. P. 112	Na_2CO_3 equimol. with 1.5% NaCl	8.15	Urine 21 B. P. 124	8.15	Urine 14 B. P. 115	8.0	Urine 7 B. P. 110	8.0	Urine 4 B. P. 100	30%	Trace No casts	Trace
6	Trace of albumin Casts	45%	8.0	25 cc. per kg 0.9% NaCl	Urine 10 B. P. 100	Na_2CO_3 equimol with 1.5% NaCl	8.2	Urine 31 B. P. 118	8.0	Urine 30 B. P. 118	Na_2CO_3 equimol. with 1.5% NaCl	8.25	Urine 28 B. P. 120	8.15	Urine 7 B. P. 110	8.1	Urine 3 B. P. 110	8.1	Urine 3 B. P. 100	40%	Trace No casts	0
7	Heavy trace albumin Casts	40%	8.0	25 cc. per kg. 0.9% NaCl	Urine 21 B. P. 132	Na_2CO_3 equimol. with 3% NaCl	8.3	Urine 28 B. P. 142	8.2	Urine 12 B. P. 130	Na_2CO_3 equimol. with 3% NaCl	8.4	Urine 12 B. P. 136	8.4	Urine 4 B. P. 120	8.3	Urine 0 B. P. 112	8.3	Urine 0 B. P. 110	20%	Trace Casts	0
8	Heavy trace albumin Casts	35%	7.9	25 cc. per kg. 0.9% NaCl	Urine 6 B. P. 110	Na_2CO_3 equimol. with 3% NaCl	8.2	Urine 14 B. P. 115	8.2	Urine 10 B. P. 120	Na_2CO_3 equimol. with 3% NaCl	8.4	Urine 5 B. P. 126	8.4	Urine 1 B. P. 118	8.4	Urine 0 B. P. 120	8.35	Urine 0 B. P. 102	Trace	Trace Casts	0
9	Trace of albumin Casts	45%	8.05	25 cc. per kg. 0.9% NaCl	Urine 14 B. P. 98	Na_2CO_3 equimol. with 3% NaCl	8.3	Urine 20 B. P. 122	8.1	Urine 26 B. P. 118	Na_2CO_3 equimol. with 3% NaCl	8.3	Urine 26 B. P. 124	8.25	Urine 4 B. P. 120	8.2	Urine 1 B. P. 105	8.2	Urine 1 in 2 min. B. P. 107	10%	Trace No casts	0
10	Trace of albumin Numerous Casts	48%	8.1	25 cc. per kg. 0.9% NaCl	Urine 18 B. P. 120	Na_2CO_3 equimol. with 3% NaCl	8.3	Urine 28 B. P. 134	8.15	Urine 36 B. P. 122	Na_2CO_3 equimol. with 3% NaCl	8.4	Urine 30 B. P. 130	8.3	Urine 12 B. P. 130	8.3	Urine 4 B. P. 121	8.3	Urine 0 B. P. 122	15%	Trace No casts	0
11	Trace of albumin Casts	56%	8.0	25 cc. per kg. 0.9% NaCl	Urine 6 B. P. 110	Na_2CO_3 equimol with 3% NaCl	8.35	Urine 18 B. P. 124	8.2	Urine 10 B. P. 112	Na_2CO_3 equimol. with 3% NaCl	8.4	Urine 14 B. P. 118	8.35	Urine 1 B. P. 100	8.2	Urine 0 B. P. 100	8.2	Urine 0 B. P. 98	Trace	Not examined	0
12	Heavy trace albumin Casts	30%	7.95	25 cc. per kg. 0.9% NaCl	Urine 3 B. P. 105	Na_2CO_3 equimol. with 3% NaCl	8.3	Urine 11 B. P. 118	8.2	Urine 5 B. P. 115	Na_2CO_3 equimol. with 3% NaCl	8.5	Urine 2 B. P. 120	8.4	Urine 0 B. P. 110	8.4	Urine 0 B. P. 105	8.35	Urine 0 B. P. 100	Trace	Trace No casts	0
13	Trace of albumin Casts	42%	8.0	25 cc. per kg. 0.9% NaCl	Urine 14 B. P. 116	Na_2CO_3 equimol. with 3% NaCl	8.25	Urine 22 B. P. 128	8.1	Urine 20 B. P. 120	Na_2CO_3 equimol. with 3% NaCl	8.35	Urine 27 B. P. 122	8.2	Urine 20 B. P. 115	8.1	Urine 16 B. P. 110	8.1	Urine 5 B. P. 110	25%	Trace No casts	0
14	Trace of albumin Casts	38%	8.05	25 cc. per kg. 0.9% NaCl	Urine 6 B. P. 120	Na_2CO_3 equimol. with 3% NaCl	8.35	Urine 18 B. P. 136	8.2	Urine 7 B. P. 110	Na_2CO_3 equimol. with 3% NaCl	8.4	Urine 10 B. P. 124	8.35	Urine 2 B. P. 120	8.3	Urine 0 B. P. 110	8.3	Urine 0 B. P. 100	10%	Trace No casts	0

2. When the naturally nephropathic animal is subjected to a second injection of such a solution there occurs a second disturbance in the acid-base equilibrium of the blood more marked than that induced by the first injection. Even though the same amount of solution per kilogram of body weight was injected, there developed in only one animal an increase in urine formation. In the remaining animals the flow of urine either remained unchanged or was reduced.

There is, following this second injection of a solution of sodium carbonate, an attempt on the part of the animals to re-establish a normal acid-base equilibrium of the blood, and as a result the reserve alkali is reduced but not to the readings normal for the respective animals.

The normal physico-chemical state of the blood is not re-established and the kidneys reflect this change in their environment by a decrease in their functional response. Urine formation is progressively lessened but not arrested. This decrease in functional response is furthermore shown by a reduction in the elimination of phenolsulphonephthalein.

3. One of the animals in this group had before the experiment a reserve alkali of the blood of 7.95. In this animal the two injections of the alkaline solution induced less disturbance in the acid-base equilibrium of the blood than was obtained in animals with a normal reserve alkali. At the termination of the experiment the reserve alkali of this animal was normal. During the course of the experiment the animal remained freely diuretic and the elimination of phenolsulphonephthalein was reduced only 8 per cent. from the normal.

4. From these observations it would appear that naturally nephropathic animals normally under the strain as a result of their kidney injury to maintain a normal acid-base equilibrium of the blood, can, following one injection of a carbonate solution equimolecular with 1.5 per cent. sodium chloride solution, readjust this equilibrium, in part by a profuse secretion of urine. When, however, such kidneys are subjected to a second strain by such an injection the mechanism through which the readjustment takes place becomes impaired, urine formation is not increased but is reduced, and the acid-base equilibrium of the blood remains disturbed. With such

a physico-chemical disturbance persisting, not only is urine formation progressively reduced, but the elimination of phenolsulphonephthalein shows a reduction which is correlated with the degree of disturbance in the acid-base equilibrium of the blood.

5. The histological changes developing in the kidney consist of cloudy swelling, edema, and vacuolation of the tubular epithelium. Such changes have constantly developed in the kidney when its blood chemical environment was sufficiently disturbed by the use of either an acid or an alkaline solution.

The Effect of a Sodium Carbonate Solution Equimolecular with a 3 Per Cent. Solution of Sodium Chloride on Renal Function and Pathology in Naturally Nephropathic Dogs.

Ten naturally nephropathic animals were used in this series of experiments. The urine from all of the animals contained both albumin and casts. The elimination of phenolsulphonephthalein by the different animals varied from a minimum output of 30 per cent. to a maximum output of 56 per cent. The reserve alkali of the blood varied from 7.9 to 8.1.

Following the development of a state of anesthesia from Gréhant's anesthetic the animals were given the usual preliminary injection of an isotonic solution of sodium chloride. The degree of diuresis obtained varied from a flow of urine of 3 drops per minute to a maximum flow of 18 drops of urine. The systolic blood pressure in the different animals varied from 98 to 120 mm. of mercury.

After an interval of half an hour the animals were given the first intravenous injection of sodium carbonate solution equimolecular with a 3 per cent. solution of sodium chloride. This resulted in an increase in the alkali reserve of the blood in all of the animals, that varied from 8.2 in the animal of Experiment 8 that had a normal alkali reserve of 7.9, to a reading of 8.35 in the animals of Experiments 11 and 14.

Following this degree of disturbance in the acid-base equilibrium of the blood, there occurred only a transitory increase in urine formation, which was not so marked as was the case in the previously described group of naturally nephropathic animals that received the weaker carbonate solution. The maximum increase in urine of 12 drops per minute developed in the animals of Experiments 11 and 14.

The introduction of this volume of fluid caused a rise in the systolic blood pressure of all of the animals. This varied from the minimum rise of 10 mm. of mercury in the animal of Experiment 7 to a maximum rise of 24 mm. of mercury in the animal of Experiment 9.

At the end of the second half hour period of the experiments all of the animals, with the exception of the animal of Experiment 8, had made some attempt to reestablish a normal acid-base equilibrium of the blood. In no instance was this equilibrium restored.

Urine formation at this early stage of the experiments was reduced in all of the animals save two, even though the systolic blood pressure was well maintained and the blood hydremic from the use of the isotonic salt solution and the solution of sodium carbonate.

In two of the animals urine formation was increased. In these animals there had occurred a more marked reduction in the alkali reserve of the blood than in the other animals of the group. In the animal of Experiment 9, the reserve alkali was reduced from 8.3 to 8.1. The normal reserve alkali for this animal was 8.05. In the animal of Experiment 10, the reserve alkali was reduced from 8.3 to 8.15. The normal alkali reserve for this animal was 8.1.

From these observations it would appear that, if the animal is able to readjust the normal acid-base equilibrium of the blood to within the range of the normal, urine formation is increased. If, however, such a readjustment cannot be made, even though the blood of the animal be hydremic and the systolic blood pressure well maintained, urine formation is reduced.

At the end of the second half hour period of the experiments the animals were given the second injection of the stronger carbonate solution. This injection induced a secondary increase in the reserve alkali of the blood which varied from 8.3 to 8.5.

There occurred a rise in the systolic blood pressure of all of the animals. The blood pressure of the different animals varied from 118 to 136 mm. of mercury.

Following these changes there was a transitory and slight increase in urine formation in two of the animals; no increase occurred in one animal, and in the remaining animals urine formation was reduced.

By the end of the fourth half-hour period of the experiments, one hour after the second injection of the carbonate solution, there was but slight change in the acid-base equilibrium of the blood. The reserve alkali readings varied from 8.2 to 8.4. Those animals in which the reserve alkali of the blood showed the least depletion toward the normal showed the greatest reduction in urine formation. The animal of Experiment 12 was anuric at this period and two of the animals, Experiments 8 and 11, were forming only 1 drop of urine per minute.

From this stage of the experiments until their termination two hours later, the changes were in general constant for all of the animals. The disturbance in the acid-base equilibrium of the blood was not adjusted. At the conclusion of the experiments the reserve alkali readings varied from 8.1 to 8.35.

Urine formation underwent a progressive reduction, so that at the end of the experiments six of the ten animals were anuric and one of the remaining animals was forming only 1 drop of urine every two minutes. The maximum urine flow was 5 drops per minute.

Those animals in which there persisted the more marked disturbance in the acid-base equilibrium of the blood were the first animals of the group to show a marked reduction in urine formation or to become anuric; while the animals in which the reserve alkali of the blood had been reduced toward the normal were the animals that formed urine to a later period in the experiments or remained diuretic until their termination.

The urine collected during the course of the experiments contained only a trace of albumin and no casts. In five of the animals the amount of albumin was distinctly less than was normal for the nephropathic animal. Diacetic acid was not present in the urine.

In all of the animals the elimination of phenolsulphonephthalein underwent a marked reduction. In three of the animals only a trace of the dye could be detected in the urine. In the remaining animals in which urine formation was sufficient to permit a determination, the elimination varied from 10 to 25 per cent. This observation is of interest when contrasted with other changes in the urine. The amount of albumin in the urine may be reduced to a mere trace and yet the elimination of phenolsulphonephthalein may be so low as not to permit a determination.

The histological changes which developed in these naturally nephropathic animals show an exaggeration of the type of change that has been described as occurring in the animals that received a weaker solution of sodium carbonate.

The same type of chronic glomerular pathology has been observed. No acute exudative or degenerative changes have developed in the glomeruli. The tubular epithelium either shows no stainable lipoid material, or it is present as minute dust-like particles.

The characteristic damage to the kidney occurs in the cells of the convoluted tubules. These cells are severely swollen, vacuolated and undergoing necrosis. Such changes are less marked in those animals that were able to effect some restoration toward the normal in the acid-base equilibrium of the blood. Fig. 4. Study II.

Conclusions Concerning the Effect of a Solution of Sodium Carbonate Equimolecular with a 3 Per Cent. Solution of Sodium Chloride on Renal Function and Pathology in Naturally Nephropathic Dogs.

1. A solution of sodium carbonate equimolecular with a 3 per cent. solution of sodium chloride is more toxic for naturally nephropathic animals than is a solution equimolecular wth a 1.5 per cent. solution of sodium chloride.

When the stronger solution is given to such animals there occurs a more marked disturbance in the acid-base equilibrium of the blood and with this greater degree of disturbance there is shown less ability on the part of the animals to deplete the reserve alkali and re-establish a normal physico-chemical state of the blood.

2. After a transitory increase in urine formation following such injections, there develops a rapid reduction in urine formation.

The persisting and marked change in the character of the blood furnished the kidney so alters its environment that its functional response is reduced very early in the experiments.

3. A second injection of such a solution intensifies this disturbance, and following it the organism shows a lessened ability to readjust its acid-base equilibrium toward the normal. Urine formation is further reduced or the animals become anuric, even though the systolic blood pressure of the animals be well maintained and the blood going to the kidney be kept in a hydremic state.

4. This inability of the kidney to functionate with such a disturbance in the acid-base equilibrium of the blood supplied, is furthermore shown by its inability to eliminate phenolsulphonephthalein.

5. The anatomical changes developing in the kidney under such a changed environment are similar in character, though more extensive in degree, to those changes previously described for the kidneys of naturally nephropathic animals that received a weaker solution of the alkali and which were able more nearly to restore the acid-base equilibrium of the blood to the normal.

General Discussion of the Effect on Renal Function and Pathology of Inducing a Disturbance in the Acid-Base Equilibrium of the Blood of Both Normal and Naturally Nephropathic Animals by the Introduction of Acid and Alkaline Solutions.

A review of the results obtained in Studies I and II in which acid and alkaline solutions were administered intravenously to both normal and naturally nephropathic animals not only shows certain variations in the quantitative response of these two types of animals to such solutions, but the observations permit certain conclusions concerning the influence of changes in the acid-base equilibrium of the blood on renal function and pathology. Probably similar changes develop in the functional units of the organism other than the kidneys. The kidney, as was pointed out earlier in these studies, was selected for these observations on account of the ease with which its functional response can be ascertained and for the reason that it is the functional unit of the animal that has most to do with maintaining a normal physico-chemical state of the blood, to which not only the kidney but all other functional units must probably adjust themselves in order to functionate in a normal manner.

When a normal solution, such as isotonic sodium chloride, is given intravenously either to normal or to naturally nephropathic animals there occurs no pronounced diuretic effect even though certain conditions are made more favorable for urine formation. Such a solution produces a more hydremic blood, the viscosity of the blood is decreased and there is a rise in systolic blood pressure.

When abnormal solutions, such as solutions of hydrochloric acid or sodium carbonate, are given to normal or to naturally nephropathic animals a change in the blood chemical environment of the animal is induced, and there is at once thrown into operation a mechanism, the kidney, which attempts by a great increase in its functional response to restore the normal physico-chemical state of the blood in so far as the normal acid-base equilibrium of the blood is concerned.

In normal animals, as such a restoration is effected, there occurs a decrease in urine formation which is not due to a renal injury induced by the solutions. At such a time, when urine formation is decreased, the elimination of phenolsulphonephthalein by the kidney is but slightly interfered with. If the use of such solutions be repeated and the acid-base equilibrium of the blood again disturbed, the kidney responds to this further change in its environment by another attempt at readjustment. In this second response even the normal kidney has been found to be inadequate. The disturbed physico-chemical state of the blood is not readjusted with sufficient rapidity or to a proper degree to enable the kidney to cope with its environment, and this lack of adjustment is expressed functionally by a decrease in urine formation or the development of an anuria, by the appearance of albumin in the urine, and by a greatly reduced elimination of phenolsulphonephthalein.

The pathological response on the part of the kidneys of both normal and naturally nephropathic dogs to such an alteration in their blood chemical environment has with one difference been the same, irrespective whether the changed environment was due to an excess of hydrogen or hydroxyl ions. The changes in the kidney can not therefore be ascribed to any specific influence of acid or alkaline solutions.

These changes consisted in edema and vacuolation of the renal epithelium and particularly of the specialized cells which line the convoluted tubules.

The necrotic changes in the epithelium varied in the different animals. Those animals that effected the least restoration toward the normal in the acid-base equilibrium of the blood were the animals in which the most marked epithelial degeneration occurred, regardless of whether an acid or an alkaline solution was employed.

The difference in the pathological response of the kidneys of these animals to acid or alkaline solutions was associated with the amount of stainable lipoid material which can be demonstrated in the renal epithelium. This difference does not depend upon the degree of the disturbance in the acid-base equilibrium of the blood, but is associated with the character of the disturbance, whether it be due to an excess of hydrogen or hydroxyl ions. If the disturbance was induced by the introduction of an acid solution, the amount of stainable lipoid material is very greatly increased in the cells of the loops of Henle and furthermore appears in the convoluted tubule epithelium. If, on the other hand, the change in the acid-base equilibrium of the blood was induced by the use of an alkaline solution, there is a decrease or absence of such stainable lipoid in the cells of the loops of Henle and in the cells of the convoluted tubules.

In the studies that have been made in naturally nephropathic animals of the disturbance in the acid-base equilibrium of the blood from the use of acid or alkaline solutions a similar type or quality of response was obtained, though the degree of disturbance was in excess of that observed in normal animals.

In six of the naturally nephropathic animals, the kidneys were unable prior to any experimental interference to maintain a normal acid-base balance of the blood. When a solution of hydrochloric acid is administered to such an animal normally under the strain as a result of the chronic nephropathy of maintaining a normal acid-base equilibrium of the blood, the solution induces a more marked disturbance in this physico-chemical state of the blood than is induced in a normal animal.

The kidney responds to this changed environment by an increase in urine formation, which in turn is not so marked as that which occurs in a normal animal. The restoration of the acid-base equilibrium of the blood is not accomplished to the same degree as occurs in normal animals. The changed environment persists, an adaptation of the kidney to this environment in terms of its functional response in not made, and urine formation is reduced. When such a solution is again introduced into a naturally nephropathic animal, there occurs a further departure from the normal in the blood chemical environment of the kidney, and urine formation is usually rapidly reduced and a state of anuria established.

When alkaline solutions are given intravenously to naturally nephropathic animals, the results obtained depend upon two factors: first, the molecular concentration of the solution; and second, whether or not at the time of the use of the solution the animal was able to maintain a normal alkali reserve of the blood.

When solutions of sodium carbonate equimolecular with a 1.5 per cent. solution of sodium chloride are administered to naturally nephropathic animals with a normal acid-base equilibrium of the blood, there occurs following the disturbance induced in this equilibrium a free diuresis. The kidney either effects a restoration of its normal blood chemical environment or a restoration is established to such an extent that the kidney remains uninjured.

When a second injection of such a solution is employed, the disturbance in the acid-base equilibrium of the blood is more marked than occurred from the first injection. The change in environment is so great that the kidney can not effect a rapid readjustment, and the inability to make this adjustment is shown by a decrease in urine formation, which may go to the extent of the establishment of a state of anuria. When, however, such a solution of sodium carbonate is given to naturally nephropathic animals with a decrease in the reserve alkali of the blood below the normal, the result is either to re-establish a normal blood chemical environment for the kidney to functionate in, or to disturb this environment to a less degree than is induced when such a solution is given to an animal with a normal acid-base equilibrium of the blood. In such animals the functional response of the kidney is improved, urine formation is more marked, and the elimination of phenolsulphonephthalein is reduced to a less extent.

The response of naturally nephropathic animals to intravenous injections of a solution of sodium carbonate equimolecular with a 3 per cent. solution of sodium chloride is of the same general character as that outlined when the weaker solution of the alkali is given to such animals. The disturbance in the acid-base equilibrium of the blood is more marked from such a solution, so that even from the first injection only a slight diuretic effect may be obtained. The blood chemical environment of the kidney remains severely altered.

When the second injection of the stronger carbonate solution is employed, with the kidney even at this early stage unable to readjust its environment, there occurs in most of the animals a further reduction in urine formation, a more marked disturbance in the physicochemical state of the blood, a very great reduction or a practical absence of the elimination of phenolsulphonephthalein, and in the majority of the animals an anuria is established.

The pathological response of the naturally nephropathic kidney to the alkaline solutions of different molecular concentration has been of the same type as has occurred from the use of acid solutions, with the exception noted in connection with the amount of stainable lipoid material that can be demonstrated in the renal epithelium. These changes have been very largely localized in the convoluted tubule epithelium, and consisted of an edema with vacuolation and necrosis of these cells. The degree to which these changes develop depends upon the duration and severity of the disturbance in the blood chemical environment of the kidney.

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DESCRIPTION OF FIGURES.

STUDY II.

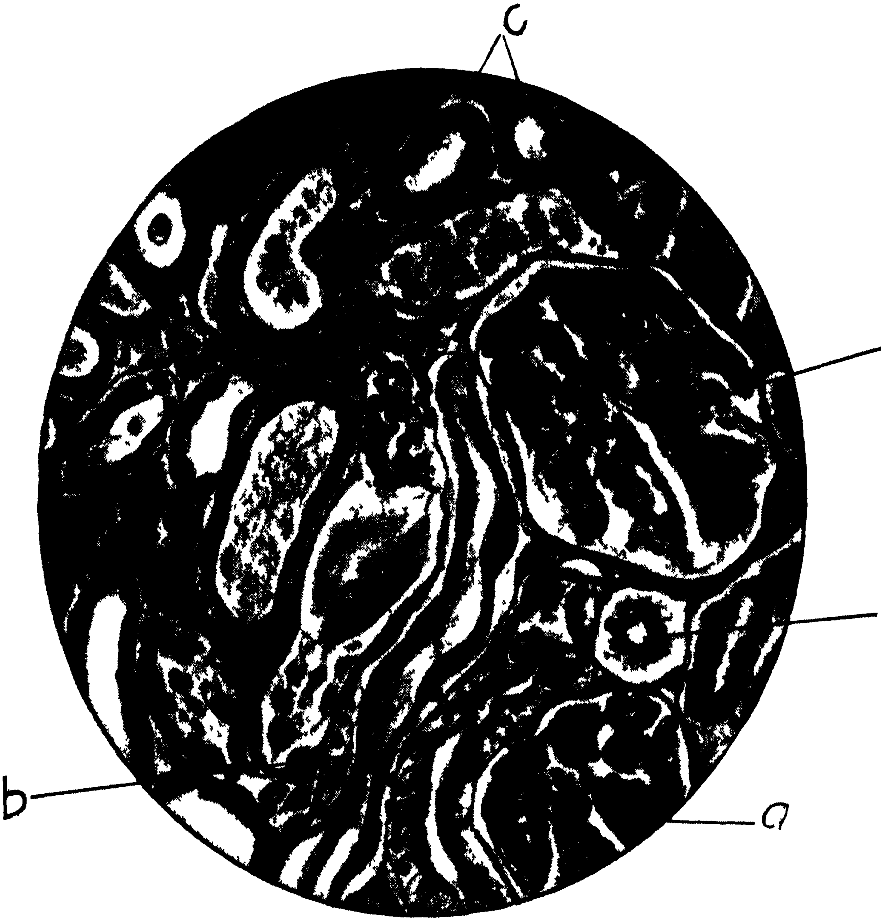

FIG. 1. Camera lucida drawing. Leitz Oc. 2, obj. 6.

The figure is from the kidney of the naturally nephropathic control animal of Experiment 1, Study II, Table 1. The animal was anesthetized by Gréhant's anesthetic for a period of four hours. With the completion of a satisfactory state of anesthesia, the animal was given intravenously 25 c.c. per kilogram of a 0.9 per cent. solution of sodium chloride. The animal did not receive either an acid or an alkaline solution during the experiment. An anesthesia of this duration with Gréhant's anesthetic in a naturally nephropathic animal has resulted in a decrease in urine formation so that at the end of the experiment only two drops of urine per minute was formed. The reserve alkali of the blood was reduced from the normal of 8.0 to 7.9. The amount of albumin in the urine was increased.

The elimination of phenolsulphonephthalein was reduced from the normal of 56 per cent. to 35 per cent.

At A, are shown glomeruli with an increase in nuclei and an obliteration of the capillary loops. The capillary mass is lobulated and adherent in places to the capsule. At B, are shown convoluted tubules with edematous epithelium. At C, are other tubules in which the swelling is less marked.

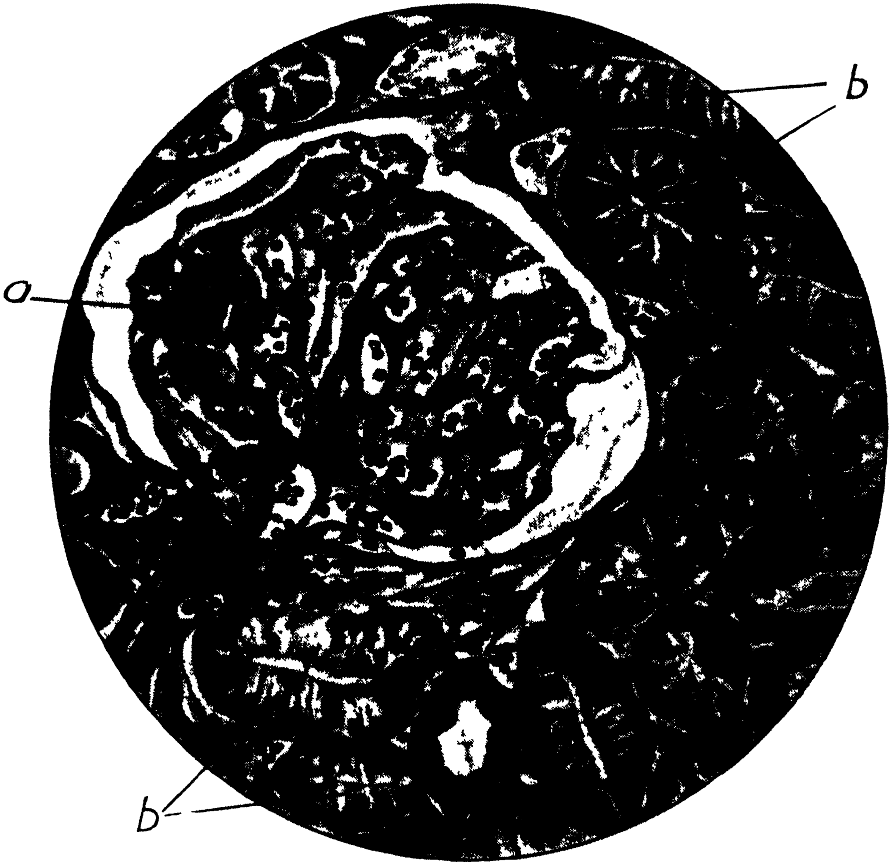

FIG. 2. Camera lucida drawing. Leitz Oc. 2, obj. 6.

The figure is from the kidney of the naturally nephropathic animal of Experiment 15, Study II, Table 1.

The animal received two injections of N/2 hydrochloric acid. The animal was unable to re-establish a normal acid-base equilibrium of the blood. The reserve alkali of the blood was reduced from the normal reading of 8.0 to 7.6. Early in the experiment a heavy precipitate of albumin appeared in the urine. Numerous casts were present. The elimination of phenolsulphonephthalein was reduced from 46 per cent. to a mere trace. Early in the experiment the animal became anuric.

At A, is shown a large glomerulus. The walls of the capillaries are greatly thickened. At one place the loops are adherent to the thickened capsule. At B, are shown convoluted tubules severely swollen, vacuolated and in an early stage of necrosis.

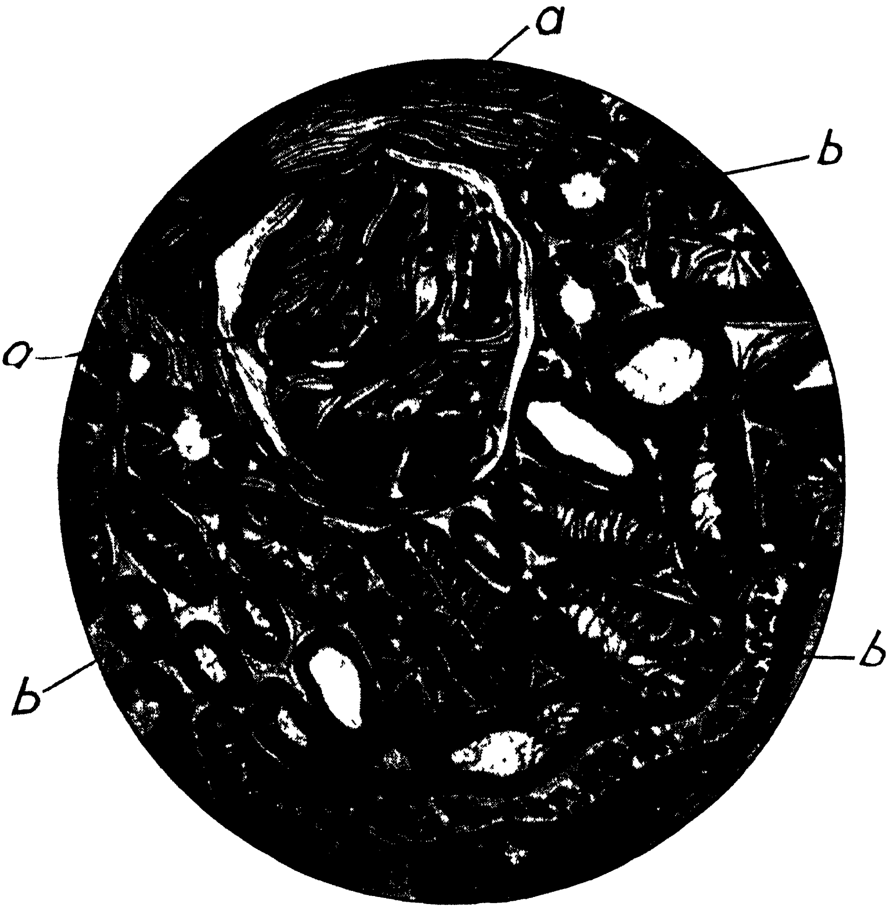

FIG. 3. Camera lucida drawing. Leitz Oc. 2, obj. 6.

The figure is from the kidney of the naturally nephropathic animal of Experiment 2, Study II, Table 2.

The animal received two intravenous injections of a solution of sodium carbonate equimolecular with a 1.5 per cent. solution of sodium chloride. The animal succeeded in a partial restoration of the acid-base equilibrium of the blood. At the termination of the experiment the reserve alkali was 8.1 as opposed to the normal reserve alkali of 8.0. The flow of urine was 5 drops per minute. The urine was free from both albumin and casts. The elimination of phenosulphonephthalein was only reduced from the normal of 42 per cent. to 38 per cent.

At A, is shown a severely fibrosed glomerulus with a thickened capsule and a periglomerular fibrosis. At B, are shown convoluted tubules with a variable amount of swelling of the cells. The nuclei in general stain well. The cells are not necrotic.

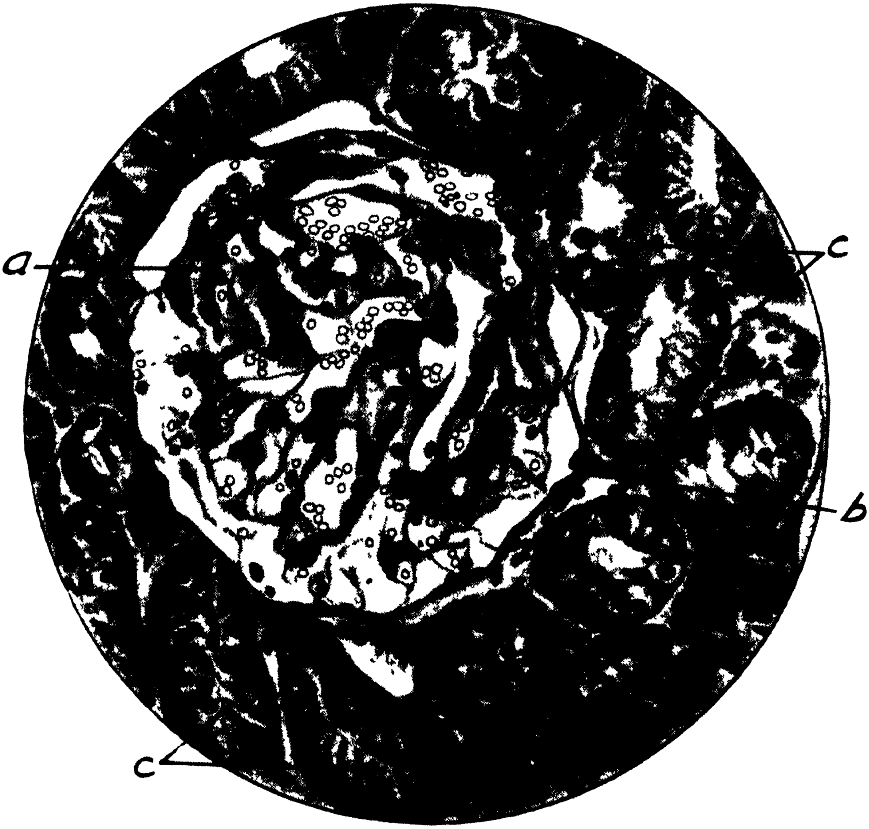

FIG. 4. Camera lucida drawing. Leitz Oc. 2, obj. 6.

The figure is from the kidney of the naturally nephropathic animal of Experiment 14, Study II, Table 2.

The animal received two intravenous injections of a solution of sodium carbonate equimolecular with a 3 per cent. solution of sodium chloride. The animal was unable to re-establish a normal acid-base equilibrium of the blood. At the termination of the experiment the reserve alkali was 8.3, as opposed to the normal reserve alkali of 8.05. The animal during the course of the experiment became anuric. Before the establishment of the anuria the urine contained albumin but no casts. Phenolsulphonephthalein appeared as a trace.

At A, is shown a large glomerulus. The capillary walls are greatly thickened. The capillary loops are adherent to the capsule which is only slightly thickened. At B, are shown convoluted tubules. The epithelium is edematous and vacuolated and shows in some tubules a well advanced necrosis.

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STUDIES CONCERNING THE INFLUENCE OF A DISTURBANCE IN THE ACID-BASE EQUILIBRIUM OF THE BLOOD ON RENAL FUNCTION AND PATHOLOGY.*

STUDY III. THE ABILITY OF AN ALKALINE SOLUTION TO PROTECT THE KIDNEY OF NORMAL AND NATURALLY NEPHROPATHIC DOGS AGAINST AN ACID SOLUTION.

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Sellards[1] in one of his earlier studies observed the value of sodium bicarbonate in the acidosis associated with the uraemia of cholera and in nephritis. At a date later than this studies[2,3] conducted in this laboratory demonstrated that the toxicity of uranium nitrate for the kidney was in part dependent upon the degree of disturbance this substance was able to induce in the acid-base equilibrium of the blood. The investigations furthermore demonstrated that if animals were protected against this disturbance in the physico-chemical state of the blood by the use of a solution of sodium carbonate, various diuretic solutions were more effective in such acutely nephropathic animals than they were in control animals that had not received the protection. More recent studies[4,5] relative to the toxic effect of the general anesthetics for the normal and naturally nephropathic kidney have shown a relationship to exist between the degree of disturbance induced by the anesthetic in the acid-base equilibrium of the blood with the toxicity of such substances for the kidney. These studies furthermore demonstrated the ability of a solution of sodium carbonate to protect the normal kidney and, to a less extent, the naturally nephropathic kidney against such an injury.

In an investigation[6] of a similar nature, the observation was made that if a solution of sodium carbonate be given acutely nephropathic animals before the commencement of an anesthetic the kidneys are

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protected against the toxic effect of the anesthetic substance. This observation was at a later date confirmed by Goto.[7] Very recently Hara[8] has shown the value of a diet which induces an alkaline urine in protecting the kidneys of rabbits against injury.

In the preceding investigations, Studies I and II, the observation has been made that when the blood chemical environment of the kidney of normal and naturally nephropathic animals was changed to a sufficient degree by a disturbance in the acid-base equilibrium of the blood, the kidney was unable to readjust its environment, a definite type of renal injury was induced, and the functional response of the kidney was decreased or suspended.

These studies have shown that the intravenous injection of 5 cc. per kilogram of a N/2 solution of hydrochloric acid in normal dogs induces such a change in the physico-chemical state of the blood that renal function is very greatly reduced. When such solutions are administered to naturally nephropathic animals, either a more marked interference with renal function occurs or an anuria develops. A further observation has been made in these experiments: that one intravenous administration of 25 cc. per kilogram of a solution of sodium carbonate equimolecular with a 1.5 per cent. solution of sodium chloride does not alter the environment of the kidney to the extent that it can not effect a readjustment of the acid-base equilibrium of the blood to within normal bounds. The use of such a solution does not decrease the functional response of the kidney.

With these observations in mind, the following investigation was undertaken to ascertain the ability of a solution of sodium carbonate equimolecular with a 1.5 per cent. solution of sodium chloride to protect both the normal and naturally nephropathic kidney against the toxic effect of a N/2 solution of hydrochloric acid.

Ten normal and eight naturally nephropathic dogs were used in these experiments. Five of the normal animals served for control experiments. Such animals were given the solution of hydrochloric acid but did not receive prior to such an injection a solution of sodium carbonate. The remaining animals were first given the alkaline solution and at a later period the acid solution.

Of the eight naturally nephropathic animals four were used for control experiments. They were given the acid solution but not the alkaline solution. The remaining animals were first given the solution of sodium carbonate and half an hour later a N/2 solution of hydrochloric acid. The results obtained in six of the normal animals and in six of the naturally nephropathic animals were included in Table I, Study I. The experimental technique employed in these experiments was identical with that used in the previous studies.

The Ability of an Alkaline Solution to Protect the Kidneys of Normal Dogs against an Acid Solution.

The preliminary study of these animals showed them to have a normal urine. The elimination of phenolsulphonephthalein varied from 60 to 81 per cent. The reserve alkali of the blood varied from 8.0 to 8.1.

The control experiments 1, 4 and 6, Table I, show the effect of a N/2 solution of hydrochloric acid in normal animals that had not had the protection of an alkaline solution. Experiments 3, 5 and 7 show the results obtained in such animals that have received an alkaline solution prior to the use of the solution of hydrochloric acid.

Following the development of an anesthesia from Gréhant's anesthetic, the animals were as usual given 25 cc. per kilogram of isotonic sodium chloride solution. The control animals of this normal group, Experiments 1, 4 and 6, developed a diuresis which varied from 14 to 21 drops of urine per minute. The systolic blood pressure varied from 100 to 121 mm. of mercury. The reserve alkali of the blood remained unchanged.

At the end of the first half-hour period of the experiments the animals were given 5 cc. per kilogram of a N/2 solution of hydrochloric acid. The use of the solution induced a reduction in the reserve alkali of the blood which varied from a minimum depletion of 7.9 to a maximum depletion of 7.85. Associated with this change in the acid-base equilibrium of the blood, it was observed that the animals became very freely diuretic. The flow of urine varied in the respective animals from 22 to 30 drops per minute. In three of the animals no attempt at a restoration of the reserve alkali of the blood was observed. In the remaining animals the depleted reserve alkali was restored toward the normal. By the end of the fourth half-hour period of the experiments the reserve alkali of the blood had increased in all of the animals, but in none did the readings return to the normal. At this stage of the experiments, when the animals had been unable to readjust their acid-base equilibrium, a marked reduction in urine formation developed which varied from an output of 14 drops of urine per minute by the animal of Experiment 6 to an output of 2 drops per minute by the animal of Experiment 1. The systolic blood pressure in these control animals varied from 100 to 124 mm. of mercury.

A study of the further course of the experiments showed no increase in the reduction of the alkali reserve of the blood. There is a continued inability on the part of the animals to restore the depleted reserve alkali to the normal. During this period urine formation decreased, so that at the termination of the experiments one of the animals was anuric. The maximum flow of urine was 2 drops per minute. The systolic blood pressure for the different animals varied from 102 to 118 mm. of mercury.

A study of the urine formed by the control animals during the experiments shows that the use of the acid solution without protection against it by the use of an alkali leads to the development of an albuminuria with casts. Diacetic acid was present in the urine of two of the animals. The elimination of phenolsulphonephthalein was reduced during the course of all of the experiments. The output of the dye in a two hour period varied from 33 to 40 per cent.

The histological study of the kidneys of the control animals failed to show any injury to the glomeruli. The epithelium, especially that of the convoluted tubules, showed cloudy swelling and edema. Vacuolation of these cells was not frequent or uniform and only occasionally were necrotic changes observed. Fig. I. Study III.

The results obtained in normal dogs protected against the acid solution by a preliminary administration of one injection of a solution of sodium carbonate are represented in Table I, by Experiments 3, 5 and 7.

Following an interval of half an hour after the development of an anesthesia and the usual intravenous injection of isotonic sodium chloride solution, these animals were given by vein 25 cc. per kilogram of a solution of sodium carbonate equimolecular with a 1.5 per cent. solution of sodium chloride.

The reserve alkali of the blood in the different animals increased from the normal readings of 8.0 to 8.1 before the experiments to readings after the use of the alkaline solution which varied from 8.15 to 8.25. The systolic blood pressure in the different animals varied from 126 to 146 mm. of mercury. Immediately following this disturbance in the acid-base equilibrium of the blood a profuse diuresis developed in all of the animals. The flow of urine varied from a minimum output of 31 drops of urine per minute by the animal of Experiment 5 to a maximum flow of urine of 63 drops per minute by the animal of Experiment 7.

At this stage of the experiments the animals were given intravenously 5 cc. per kilogram of a N/2 solution of hydrochloric acid. Such injections resulted in a sudden reduction of the alkali reserve of the blood and in two of the animals were followed by a reestablishment of the normal acid-base equilibrium. Following this change, at the end of the second half-hour of the experiments, the animals continued freely diuretic even though the systolic blood pressure had undergone a reduction in all but two of the animals.

From this stage of the experiments until their termination at the end of the four hour period the results have been very uniform in the different animals. As the experiments progress there occurs a gradual reduction in urine formation. None of the animals became anuric. At the end of the experimental period the flow of urine in the different animals has varied from 11 to 18 drops per minute. The reserve alkali of the blood at the end of the experiments has been within the normal for all of the animals. The reserve alkali has varied from 8.0 to 8.05.

Urine collected during the experiments has been variable as to the presence of albumin and casts. Two of the animals had a urine which showed a trace of albumin but no casts. The urine from the remaining animals was free from both albumin and casts. Diacetic acid was not present.

The elimination of phenolsulphonephthalein was reduced from the normal readings obtained prior to the experiments, but the reduction was much less than occurred in the control animals that did not receive an alkali. The elimination of the dye by the different animals varied from a minimum output of 55 per cent. to a maximum output of 65 per cent.

The histological changes in the kidneys of these animals that have been protected against the toxic effect of a solution of hydrochloric acid by the use of a solution of sodium carbonate are negative in character when compared with the results obtained in the control group of animals. The glomeruli appear normal. The tubular epithelium is shrunken. The cytoplasm stains well and shows a moderate degree of granulation. Vacuolation and necrosis of the cells were not observed. Fig. 2. Study III.

Conclusions Concerning the Ability of an Alkaline Solution to Protect the Kidney of Normal Dogs against an Acid Solution.

1. The intravenous injection of 5 cc. per kilogram of a N/2 solution of hydrochloric acid in normal dogs induces a disturbance in the acid-base equilibrium of the blood which the animal is unable to readjust in a four hour period.

2. Associated with this disturbance in the physico-chemical state of the blood, urine formation is gradually decreased or the animals become anuric. Albumin and casts appear in the urine and the elimination of phenolsulphonephthalein is markedly reduced. The kidneys of such animals show the usual histological changes common to such a disturbance in their environment. The epithelium of the tubules is edematous and vacuolated and more rarely shows an early necrosis.

3. When normal animals are given 25 cc. per kilogram of a solution of sodium carbonate equimolecular with a 1.5 per cent. solution of sodium chloride and at a later period of the experiment given a similar amount per kilogram of a solution of hydrochloric acid as was given to the control animals, the animals receiving the alkaline solution are in part protected against the toxic effect of the acid solution.

4. The use of such an alkaline solution decreases the degree of disturbance in the acid-base equilibrium of the blood which is induced by an acid solution. Such animals are able to restore the acid-base equilibrium of the blood to within normal readings. With

STUDY III. TABLE 1.

The Ability of an Alkaline Solution to Protect the Kidney of Normal and Naturally Nephropathic Dogs against an Acid Solution.

Number of experiment	Urine	Phthalein 2 hour period	R.pH	Gréhant's anesthetic 60%	Urine per minute Bl. Pres. mm. Hg	Alkaline solution 25 cc. per kg.	R.pH end of 1st half hour	Urine per minute Bl. Pres. mm. Hg	Acid solution	R.pH end of 2nd half hour	Urine per minute Bl. Pres. mm. Hg	R.pH end of 3rd half hour	Urine per minute Bl. Pres. mm. Hg	R.pH end of 4th half hour	Urine per minute Bl. Pres. mm. Hg	R.pH end of 3rd hour	Urine per minute Bl. Pres. mm. Hg	R.pH end of 4th hour	Urine per minute Bl. Pres. mm. Hg	Phthalein 2 hour period	Albumin and casts	Diacetic acid
1 Control Normal animal	Normal	76%	8.0	25 cc. per kg. 0.9% NaCl	Urine 14 B. P. 100	0	8.0	Urine 18 B. P. 111	5 cc. per kg. N/2 HCl	7.85	Urine 22 B. P. 114	7.9	Urine 10 B. P. 110	7.9	Urine 2 B. P. 100	7.9	Urine 2 B. P. 100	7.9	Urine 2 B. P. 102	34%	Albumin Casts	Present
3 Normal animal Alkali	Normal	74%	8.0	25 cc. per kg. 0.9% NaCl	Urine 10 B. P. 114	Na_2CO_3 equimol. with 1.5% NaCl	8.15	Urine 55 B. P. 126	5 cc. per kg. N/2 HCl	8.15	Urine 42 B. P. 130	8.15	Urine 19 B. P. 120	8.1	Urine 12 B. P. 120	8.05	Urine 8 B. P. 110	8.05	Urine 12 B. P. 121	61%	Trace of albumin No casts	0
4 Control Normal animal	Normal	64%	8.1	25 cc. per kg. 0.9% NaCl	Urine 17 B. P. 114	0	8.1	Urine 24 B. P. 131	5 cc. per kg. N/2 HCl	7.9	Urine 30 B. P. 133	7.9	Urine 16 B. P. 130	8.0	Urine 4 B. P. 124	7.9	Urine 1 B. P. 120	7.9	Urine 1 in 3 min. B. P. 118	40%	Trace of albumin Casts	Not made
5 Normal animal Alkali	Normal	81%	8.0	25 cc. per kg. 0.9% NaCl	Urine 8 B. P. 130	Na_2CO_3 equimol. with 1.5% NaCl	8.25	Urine 31 B. P. 142	5 cc. per kg. N/2 HCl	8.1	Urine 37 B. P. 142	8.1	Urine 30 B. P. 130	8.05	Urine 21 B. P. 130	8.05	Urine 14 B. P. 120	8.05	Urine 12 B. P. 116	65%	No albumin No casts	0
6 Control Normal animal	Normal	60%	8.1	25 cc. per kg. 0.9% NaCl	Urine 21 B. P. 121	0	8.1	Urine 18 B. P. 115	5 cc. per kg. N/2 HCl	7.9	Urine 26 B. P. 110	7.9	Urine 14 B. P. 112	8.0	Urine 14 B. P. 118	7.95	Urine 2 B. P. 110	7.95	Urine 0 B. P. 112	33%	Trace of albumin No casts	Present
7 Normal animal Alkali	Normal	71%	8.1	25 cc. per kg. 0.9% NaCl	Urine 4 B. P. 132	Na_2CO_3 equimol. with 1.5% NaCl	8.25	Urine 63 B. P. 146	5 cc. per kg. N/2 HCl	8.1	Urine 68 B. P. 140	8.1	Urine 42 B. P. 130	8.1	Urine 22 B. P. 122	8.0	Urine 20 B. P. 120	8.0	Urine 18 B. P. 126	55%	No albumin No casts	0
1 Control Nephropathic animal	Albumin Casts Glucose	52%	8.0	25 cc. per kg. 0.9% NaCl	Urine 11 B. P. 105	0	8.0	Urine 4 B. P. 112	5 cc. per kg. N/2 HCl	7.9	Urine 6 B. P. 115	7.9	Urine 0 B. P. 121	7.8	Urine 0 B. P. 110	7.8	Urine 0 B. P. 105	7.75	Urine 0 B. P. 100	Trace	Not made	Not made
4 Nephropathic animal Alkali	Albumin Casts Diacetic acid	42%	8.0	25 cc. per kg. 0.9% NaCl	Urine 7 B. P. 112	Na_2CO_3 equimol. with 1.5% NaCl	8.15	Urine 26 B. P. 128	5 cc. per kg. N/2 HCl	8.0	Urine 20 B. P. 120	7.9	Urine 12 B. P. 116	7.95	Urine 14 B. P. 112	7.95	Urine 11 B. P. 105	7.95	Urine 8 B. P. 105	30%	Trace of albumin No casts	Present
5 Control Nephropathic animal	Albumin Casts	48%	7.95	25 cc. per kg. 0.9% NaCl	Urine 4 B. P. 127	0	7.9	Urine 1 in 2 min. B. P. 130	5 cc. per kg. N/2 HCl	7.8	Urine 0 B. P. 130	7.8	Urine 0 B. P. 124	7.7	Urine 0 B. P. 120	7.7	Urine 0 B. P. 105	7.7	Urine 0 B. P. 100	Not made	Not made	Not made
6 Nephropathic animal Alkali	Trace of albumin Very few casts	40%	7.9	25 cc. per kg. 0.9% NaCl	Urine 4 B. P. 130	Na_2CO_3 equimol. with 1.5% NaCl	8.1	Urine 27 B. P. 138	5 cc. per kg. N/2 HCl	8.0	Urine 24 B. P. 130	8.0	Urine 18 B. P. 130	8.0	Urine 20 B. P. 122	7.9	Urine 12 B. P. 120	7.9	Urine 14 B. P. 115	36%	Trace of albumin No casts	Present
8 Control Nephropathic animal	Albumin Casts Glucose	34%	8.0	25 cc. per kg. 0.9% NaCl	Urine 18 B. P. 118	0	8.0	Urine 19 B. P. 120	5 cc. per kg. N/2 HCl	7.85	Urine 8 B. P. 118	7.85	Urine 4 B. P. 112	7.8	Urine 1 B. P. 120	7.8	Urine 0 B. P. 110	7.8	Urine 0 B. P. 105	Trace	Trace of albumin Casts	Not made
9 Nephropathic animal Alkali	Albumin Casts	38%	8.0	25 cc. per kg. 0.9% NaCl	Urine 7 B. P. 126	Na_2CO_3 equimol. with 1.5% NaCl	8.2	Urine 31 B. P. 136	5 cc. per kg. N/2 HCl	8.1	Urine 32 B. P. 136	8.1	Urine 24 B. P. 130	8.0	Urine 20 B. P. 130	8.0	Urine 8 B. P. 120	8.0	Urine 8 B. P. 120	22%	Trace of albumin No casts	Present

this lack of departure in the blood chemical environment of the kidney from the normal, urine formation continues throughout the experiments, either no albumin appears in the urine or it occasionally appears as a trace. The elimination of phenolsulphonephthalein is but slightly reduced.

5. The histological study of the kidneys of such animals shows anatomical evidence of protection. The epithelium of the tubules is not edematous or vacuolated as is the case with such cells that have been subjected to a changed environment from the use of an acid solution without the protection of an alkali.

The Ability of an Alkaline Solution to Protect the Kidney of Naturally Nephropathic Dogs against an Acid Solution.

Eight naturally nephropathic animals were used in these experiments. The urine from all of the animals contained both albumin and casts. The elimination of phenolsulphonephthalein varied in the respective animals from 34 to 52 per cent. The reserve alkali of the blood varied from 7.9 to 8.05.

The results obtained from a study of this group of animals are represented by six experiments included in Table I, Study III. The animals of Experiments 1, 5 and 8 served in the capacity of controls. These dogs received one injection of N/2 solution of hydrochloric acid without a preliminary injection of an alkaline solution. The animals of Experiments 4, 6 and 9 were first given intravenously a solution of sodium carbonate and at a later period in the experiments an acid solution.

Following the development of an anesthesia from Gréhant's anesthetic the naturally nephropathic animals were given intravenously 25 cc. per kilogram of a 0.9 per cent. solution of sodium chloride. Urine formation by these animals varied from 4 to 18 drops per minute. The naturally nephropathic animals were less responsive to the diuretic effect of such a solution than was the case with the former group of normal animals. The systolic blood pressure in the naturally nephropathic dogs has varied from 105 to 130 mm. of mercury.

The control animals of this group were now given 5 cc. per kilogram of a N/2 solution of hydrochloric acid.

The reserve alkali of the blood was reduced to readings which varied from 7.8 to 7.9. In the animal of Experiment 1, in which the reserve alkali was only reduced from the normal of 8.0 to 7.9, there was an initial increase in urine formation from 4 drops per minute to 6 drops per minute. In the animal of Experiment 5, in which the reserve alkali was reduced from 7.95 to 7.8, the animal became anuric. In the remaining animals urine formation was reduced. There was no attempt by any of the naturally nephropathic animals to restore the normal acid-base equilibrium of the blood. On the contrary, during the course of the experiments the reserve alkali of the blood underwent a progressive depletion. At the termination of the experiments the reserve alkali readings for the different animals varied from the low reading of 7.7 to 7.8.

Associated with the continuation of this disturbance in the physico-chemical state of the blood, urine formation rapidly decreased in all of the animals so that by the end of the third hour of the experiments all of the animals were anuric. At this period of the establishment of an anuria the systolic blood pressure for the respective animals varied from 105 to 110 mm. of mercury.

The results obtained in this control group of naturally nephropathic animals differ only in degree from those obtained in the group of control normal animals. The normal animals that received a solution of hydrochloric acid were able either to restore the acid-base equilibrium of the blood toward the normal or to maintain it at a reading not below 7.9. These animals showed a reduction in urine formation. With this degree of disturbance in the environment of the kidney, this unit was still able to functionate to a lessened degree. In the naturally nephropathic animals the use of such an acid solution causes a greater disturbance in the environment of the kidney, and the primarily damaged organ is unable to establish through increased function a restoration of the normal physico-chemical state of the blood. This inability is expressed by a rapid decrease in function and finally by its arrest.

The urine collected during these experiments contained both albumin and casts. The elimination of phenolsulphonephthalein was so greatly reduced that a quantitative determination of the output was impossible. The time of the appearance of the dye was greatly delayed, and its output was only a trace.

The histological study of the control naturally nephropathic animals shows in general the same type of chronic glomerular pathology described in the previous studies. The glomeruli show no evidence of acute degenerative changes. The tubular epithelium of the kidney is the tissue which shows the effect of the physico-chemical change that the acid solution has induced in the blood. These cells, and especially those of the convoluted tubules, show a marked edema and vacuolation and the cells of many of the tubules show an advanced necrosis. Fig. 3. Study III.

The results obtained in naturally nephropathic dogs protected against an acid solution by the preliminary administration of one injection of a solution of sodium carbonate are represented in Table I, by Experiments 4, 6 and 9.

Following the development of a state of anesthesia, the animals were given intravenously the usual solution of isotonic sodium chloride. Urine formation by the different animals varied from 4 to 7 drops per minute. The systolic blood pressure varied from 112 to 130 mm. of mercury. Determinations of the reserve alkali of the blood varied from 7.9 to 8.0.

At the end of the first half-hour period of the experiments the animals were given 25 cc. per kilogram of a solution of sodium carbonate equimolecular with a 1.5 per cent. solution of sodium chloride. The reserve alkali of the blood was increased in all of the animals, but the degree of disturbance was not so marked as was the case in the animals with a normal alkali reserve. These determinations for the group of naturally nephropathic animals following the use of the alkali varied from a reading of 8.1 in the animal of Experiment 6 to a reading of 8.2 in the animal of Experiment 9.

Associated with this change in the acid-base equilibrium of the blood, urine formation increased in all of the animals. The degree of increase was not so great as was the case with the normal animals. The flow of urine varied from a minimum output of 26 drops per minute by the animal of Experiment 4 to a maximum output of 31 drops by the animal of Experiment 9. The systolic blood pressure in the different animals varied from 128 to 138 mm. of mercury.

At this stage of the experiments the animals were given intravenously 5 cc. per kilogram of a N/2 solution of hydrochloric acid. The reserve alkali of the blood was reduced in all of the animals by such an injection, but not to a point below the normal. The alkali reserve readings for the respective animals varied from 8.0 to 8.1. Associated with a failure of the acid solution to reduce the alkali reserve of the blood below the normal, urine formation continued and in only two of the animals was there any reduction in urine formation. At this stage of the experiments with the control animals that had not received an alkaline solution urine formation was reduced, or the animals had become anuric.

During the remainder of the experiments, these naturally nephropathic animals that had received the solution of sodium carbonate continued to form urine. At the conclusion of the experiments urine formation by the different animals varied from 8 to 14 drops per minute.

As the experiments progressed the reserve alkali of the blood underwent a reduction. This change was much less marked than was the case with the control animals of the group that had not received the alkaline solution. The reserve alkali determinations at the end of the experiments varied from 7.9 to 8.0. The systolic blood pressure in the different animals varied from 105 to 120 mm. of mercury. Urine collected during the course of the experiments showed a trace of albumin and no casts. Diacetic acid was present in the urine of three of the animals.

The elimination of phenolsulphonephthalein was reduced to a greater extent than was the case with the normal animals protected by the use of an alkali, but the elimination was greater than the elimination by the naturally nephropathic animals that did not have this protection. The output of the dye in a two hour period varied from 22 to 30 per cent.

A study of the kidneys of this group of naturally nephropathic animals that received an alkaline solution prior to the use of a solution of hydrochloric acid shows anatomical evidence of protection in that there is less edema and vacuolation of the epithelium, and necrotic changes in these cells are rarely observed. Fig. 4. Study III.

Conclusions Concerning the Ability of an Alkaline Solution to Protect the Kidneys of Naturally Nephropathic Dogs against an Acid Solution.

1. The naturally nephropathic kidney is more susceptible to the toxic effect of a N/2 solution of hydrochloric acid than is the normal kidney. Such a solution in naturally nephropathic animals induces a more marked disturbance in the acid-base equilibrium of the blood. The naturally nephropathic animal is unable to readjust this disturbance and establish a normal physico-chemical state of the blood.

2. As a result of the persistence of such a disturbed environment renal function is rapidly reduced, albumin and casts increase in the urine, the elimination of phenolsulphonephthalein is delayed and decreased in its output to a trace. The animals become anuric.

3. The intravenous injection of a solution of sodium carbonate equimolecular with a 1.5 per cent. solution of sodium chloride before the use of such an acid solution confers a partial protection to the kidneys in such animals. The protection is shown by the naturally nephropathic animals being more nearly able to maintain a normal acid-base equilibrium of the blood. With the physico-chemical state of the blood more nearly approaching the normal, the naturally nephropathic animals that have received the alkaline solution continue to form urine until the termination of the experiments. The urine from such protected animals has contained only a trace of albumin and no casts. The elimination of phenolsulphonephthalein is in excess of that obtained from the animals without the protection. The histological evidence of injury to the kidney is less marked than is the case in naturally nephropathic animals that have not been protected by the use of an alkaline solution.

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STUDY III.

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2. MacNider, Wm. deB. *J. Exp. Med.*, 26, 1917, 1.
3. MacNider, Wm. deB. *J. Exp. Med.*, 26, 1917, 19
4. MacNider, Wm. deB. *J. Exp. Med.*, 28, 1918, 501.
5. MacNider, Wm. deB. *J. Exp. Med.*, 28, 1918, 517.
6. MacNider, Wm. deB. *J. Exp. Med.*, 23, 1916, 171.
7. Goto, R. *J. Exp. Med.*, 27, 1918, 413.
8. Hara, M. *Mitt. a. d. med. Fak. k. Univ. Tokio*, 25, 1920, 1.

DESCRIPTION OF FIGURES.

STUDY III.

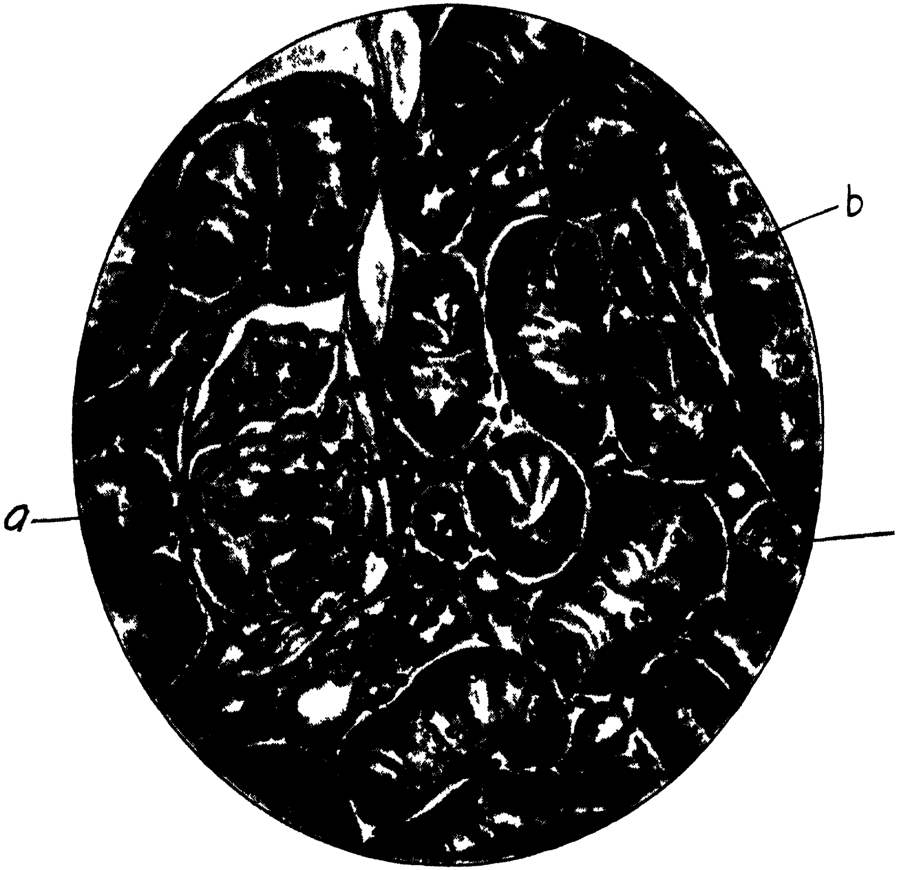

FIG. 1. Camera lucida drawing. Leitz Oc. 2, obj 6

The figure is from the normal control animal of Experiment 1, Study III, Table I.

The animal was given 5 cc. per kilogram of a N/2 solution of hydrochloric acid. There developed a marked disturbance in the acid-base equilibrium of the blood and a progressive reduction in urine formation. At the termination of the experiment the animal was forming only 2 drops of urine per minute. The reserve alkali at the end of the experiment was 7 9 The elimination of phenolsulphonephthalein was reduced from the normal of 76 per cent. to 34 per cent. Albumin and casts were present in the urine

At A, is shown a normal glomerulus At B, are shown convoluted tubules with the epithelium severely swollen. Vacuolation and necrosis are occasionally seen.

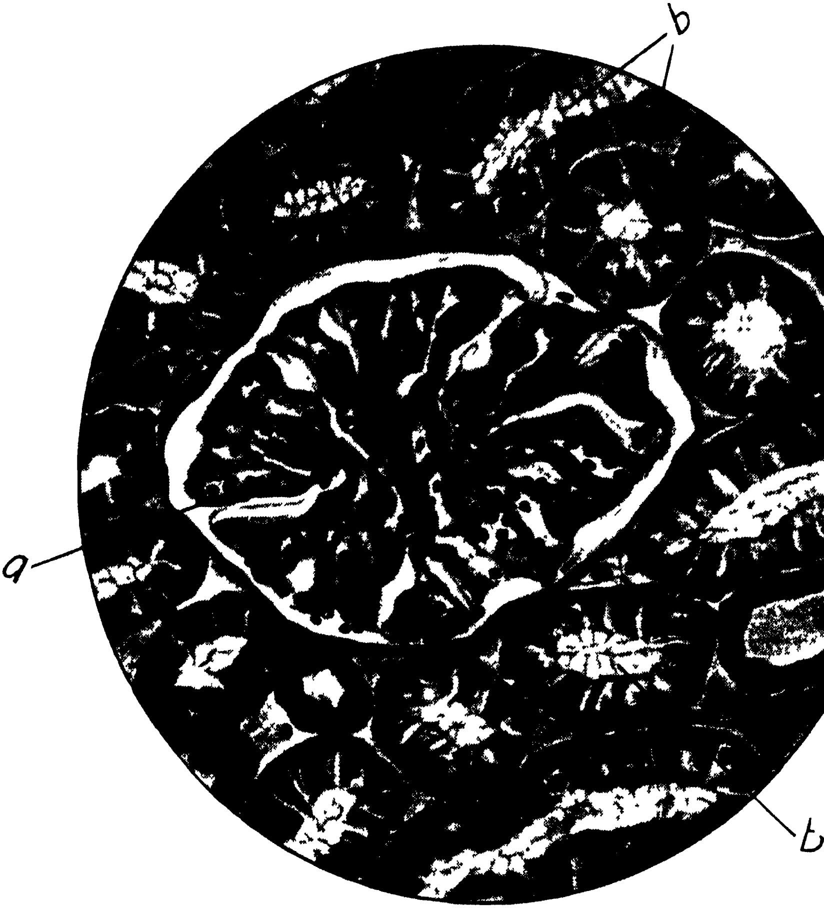

Fig. 2 Camera lucida drawing. Leitz Oc. 2, obj 6.

The figure is from the normal animal of Experiment 5, Study III, Table I.

The animal was protected against the toxic effect of a N 2 solution of hydrochloric acid by a preliminary intravenous injection of 25 cc per kilogram of a solution of sodium carbonate equimolecular with a 1 5 per cent solution of sodium chloride. The acid base equilibrium of the blood was well maintained throughout the experiment The reserve alkali of the blood at the conclusion of the experiment was 8.05 as opposed to the normal alkali reserve of 8.1. The animal was freely diuretic during the experiment. The flow of urine at the end of the experiment was 12 drops per minute Neither albumin or casts were present in the urine. The elimination of phenolsulphonephthalein was only reduced from the normal output of 81 per cent to 65 per cent

At A, is shown a normal glomerulus which fills the capsular space. At B, are shown convoluted tubules with a shrunken epithelium which stains well. The nuclei are hyperchromatic.

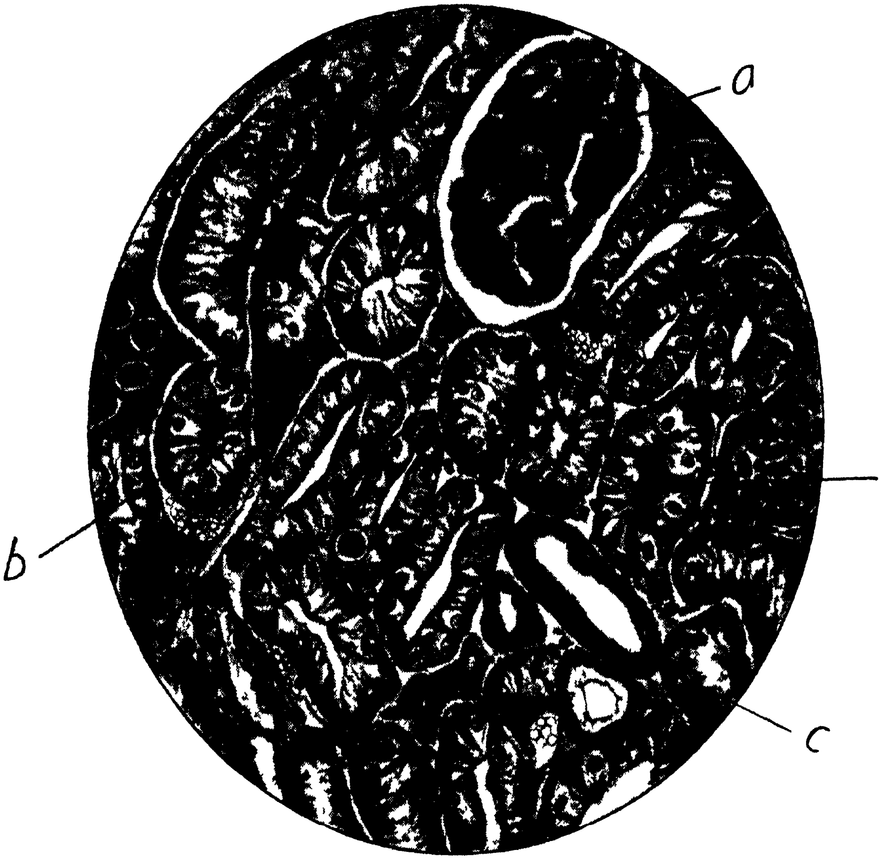

FIG. 3. Camera lucida drawing. Leitz Oc. 2, obj 6.

The figure is from the control naturally nephropathic animal of Experiment 1, Study III, Table I.

The animal was given intravenously one injection of 5 cc per kilogram of a N/2 solution of hydrochloric acid. There developed a marked disturbance in the acid-base equilibrium of the blood and a rapid reduction in urine formation. The reserve alkali of the blood at the termination the experiment was 7.75 as opposed to the normal reading of 8.0. At the end of the experiment the animal was anuric. During the experiment the elimination of phenolsulphonephthalein was reduced from the normal of 52 per cent. to a trace.

At A, is shown a glomerulus with an increase in endothelial nuclei and a matting together of the capillary loops. At B, are shown convoluted tubules in an advanced stage of edema and vacuolation. Necrosis of the epithelium is well marked. At C, are shown collecting tubules in which these degenerative changes are less pronounced.

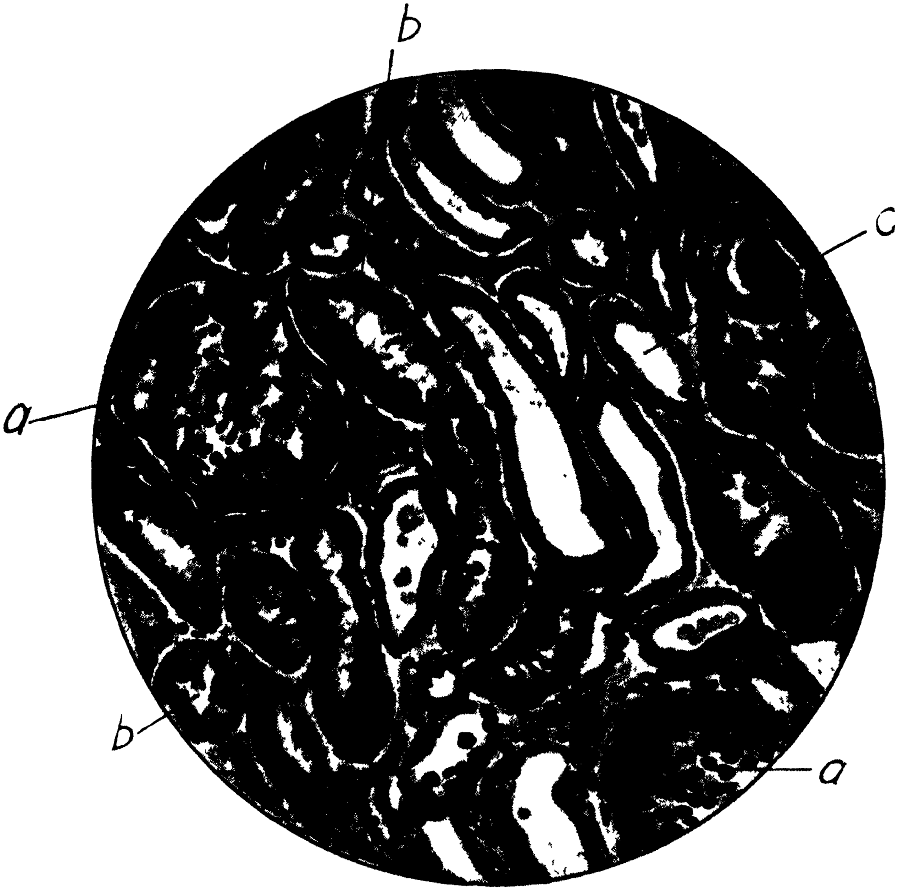

FIG 4 Camera lucida drawing. Leitz Oc. 2, obj. 6

The figure is from the unprotected naturally nephropathic animal of Experiment 9, Study III, Table I.

The animal was in part protected against the toxic effect of a N 2 solution of hydrochloric acid by a preliminary intravenous injection of 25 cc per kilogram of a solution of sodium carbonate equimolecular with a 1.5 per cent. solution of sodium chloride. The normal acid-base equilibrium of the blood was imperfectly maintained during the experiment At the end of the experiment the reserve alkali was 8.0 as opposed to the normal reading of 8.05 The animal was diuretic throughout the experiment and at its termination was forming 8 drops of urine per minute. The urine contained a trace of albumin but no casts The elimination of phenolsulphonephthalein was reduced from the normal of 38 per cent. to 22 per cent.

At A, are shown glomeruli in an early stage of an intracapillary fibrosis. The capsules of the glomeruli are thickened. At B, are shown convoluted tubules in which there is an early edema. Vacuolation and necrosis of the epithelium are rarely seen. At C, are shown the tubules of the loops of Henle which show to a less extent these changes of degeneration.

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ON THE ANTIGENIC PROPERTIES OF HEMOGLOBIN.*

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The antigenic properties of hemoglobin[1] are of interest because there is available in the form of the oxy-compound a crystalline substance which, unlike most other animal proteins, appears to be a chemical individual and can be tested for purity by chemical methods. For these reasons much work has been done on the subject, but the conclusions reached have been conflicting.

Leblanc,[2] Ide,[3] and Demees[4] were the first to report the immunization of animals with hemoglobin. Ide's antiserum was hemolytic in addition, but his pupil Demees, by purifying his hemoglobin more thoroughly, succeeded in obtaining a serum which precipitated hemoglobin solutions and did not cause hemolysis. Thorough purification is important since the blood corpuscles contain substances of stronger antigenic power (globulins, stromata) than hemoglobin (Ide). The precipitins obtained by Demees were not bound by intact red cells, and were produced only by intensive treatment of the animals.

The question of the species-specificity of hemoglobin was investigated by Thomsen[5] with the aid of the anaphylactic reaction. Most of his work was carried on with dissolved corpuscles, though he made a few experiments with the crystalline hemoglobin of two species Similar experiments were made by Bradley and Sansum.[6] Guinea pigs sensitized with dog hemoglobin reacted strongly (but not with acute death) to solutions of dog red cells, less markedly to those of the cat, pig, and turtle, and not at all to the blood of a number of other animals.

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* Seventeenth paper on antigens.

[1] The word hemoglobin, in this communication, is used in the generic sense, including oxyhemoglobin and its immediate derivatives.

[2] Leblanc, A., *La Cellule*, 1901, xviii, 337.

[3] Ide, M., *La Cellule*, 1902, xx, 263.

[4] Demees, O., *La Cellule*, 1907, xxiv, 423.

[5] Thomsen, O., *Z. Immunitätsforsch.*, *Orig.*, 1909, iii, 539. *Cf.* Schittenhelm, A., and Weichardt, W., *Z. Immunitätsforsch.*, *Orig.*, 1912, xiv, 609. Pfeiffer, H., and Mita, S., *Z. Immunitätsforsch.*, *Orig.*, 1910, vi.

[6] Bradley, H. C., and Sansum, W. D., *J. Biol. Chem.*, 1914, xviii, 497.

As there is no reason to believe that the hematin in the hemoglobin molecule of various species is different, and it is at least certain that there are not many different hematins, the differences in the antigenic properties of the different hemoglobins would appear to be due to the globin, or the protein part of the molecule. The serological behavior of globin was studied by Browning and Wilson;[7] also by Gay and Robertson.[8] The former obtained, in addition, an antihemoglobin serum and found that an immune serum against guinea pig globin fixed the complement strongly with the corresponding antigen, and reacted only weakly with rabbit globin and not at all with ox globin. In a second paper Browning and Robertson report that ox globin antiserum reacts, in addition, with the globins of the goat, guinea pig, and duck, but not with that of the rabbit They write: "Thus while evidence of species-specificity exists in certain cases, there is also a wide, though not universal, community of antigenic properties shared by the globin of widely separate animal species."

In contrast to the positive results cited above, Ford and Halsey[9] were unable to produce either antibody formation or anaphylaxis by injecting repeatedly recrystallized hemoglobin. Similar negative results with both hemoglobin and globin were reported by Schmidt and Bennett,[10, 11] using hemoglobin which had been carefully purified by various methods. They therefore concluded that hemoglobin is a non-antigenic substance and that the positive findings of previous workers were possibly due to the impurity of the hemoglobin or globin used. Subsequent to their work positive findings were again reported by Fujiwara [12]

Differences between the various antigens contained in the blood corpuscles have been investigated by Klein, Leers, Levene, Chodat, Steward, and Fujiwara.

The solution of the question of the antigenic or non-antigenic nature of hemoglobin is of considerable general interest, since if it were really impossible to produce antibodies with hemoglobin, doubts might arise as to the antigenic properties of absolutely pure proteins in general. It would seem far more reasonable, however, to view such behavior as pointing to a relationship between the constitution of individual proteins and their antigenic action. Moreover, the determination of the species-specificity of hemoglobin antibodies, if they really exist, would have importance as throwing light on the general problem of how species-specificity is manifested in the individual proteins of the animal organism. It is from this standpoint that we have taken up the matter, and a preliminary note has been published in which this phase of the problem is discussed more thoroughly.[13] It has developed that antibodies can be obtained with hemoglobin, although less readily than with most other proteins, and that these antibodies are species-specific to a high degree.

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[7] Browning, C. H., and Wilson, G. H., *J. Path. and Bact.*, 1909, xiv, 174; *J. Immunol.*, 1920, v, 417.

[8] Gay, F. P., and Robertson, T. B., *J. Exp. Med.*, 1913, xvii, 535.

[9] Ford, W. W., and Halsey, J. T., *J. Med. Research*, 1904, xi, 403.

[10] Schmidt, C. L. A., and Bennett, C. B., *J. Infect. Dis.*, 1919, xxv, 207. Schmidt, C. L. A., *Univ. Calif. Pub.*, 1916, ii, 157.

[11] Bennett, C. B., and Schmidt, C. L. A., *J. Immunol.*, 1919, iv, 29. *Cf.* Gay, F. P., and Robertson, T. B., *J. Exp. Med.*, 1913, xvii, 535.

[12] Fujiwara, K., *Mitt. med. Ges. Tokyo*, 1920, xxxiv, No. 23.

After the publication of the preliminary note, and after the completion of the present work, a communication by Higashi[14] was received in which the author's conclusions coincide essentially with our own. On the other hand, negative results were published by Depla.[15]

Recently, Hektoen and Schulhof[16] reported the production of antibodies with blood extracts and hemoglobin solutions, concluding that hemoglobin is a species-specific antigen. Contradictory in a certain measure, however, is their finding that there was no appreciable diminution of precipitable substance after fission of the hemoglobin into hematin and globin and removal of most of the split products. Their conclusion is: "While the precipitinogens in extracts of red corpuscles and in hemoglobin may exist independently of hemoglobin after treatment with acids, they ordinarily are attached closely to the hemoglobin molecule, not being removed or diminished in proportion to the amount of hemoglobin by repeated crystallization or by treatment with aluminium cream, the antigen being apparently either closely adsorbed to the hemoglobin molecule or forming a part of it which can be split off by acids." We shall discuss this conclusion further on in our paper.

Preparation of Oxyhemoglobin and Immunization of the Animals.

In the work reported in this paper horse hemoglobin only was used for immunization.

The preliminary experiments referred to above were carried out with a preparation (A) made as follows, according to the methods of Ide[3] and Demees.[4]

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[13] Landsteiner, K., *Kon. Akad. van Wetensch. te Amsterdam*, 1921, xxix, 1029.

[14] Higashi, S., *J. Biochem.* (Japan), 1923, ii, 315. *Cf. J. Tokyo Med. Soc.*, 1921, xxxv, No. 9.

[15] Depla, H., *Compt. rend. Soc. biol.*, 1922, lxxxvii, 383. *Cf.* Chodat, F., *Compt. rend. Soc. biol.*, 1921, lxxxv, 735.

[16] Hektoen, L., and Schulhof, K., *J. Infect. Dis.*, 1922, xxxi, 32.

Defibrinated horse blood was centrifuged, washed five times, and dissolved in a volume of water equal to twice the amount of blood used. Ether was added, and the mixture allowed to stand in the ice box for 1 or 2 days, with occasional stirring. After decantation from any sediment, the solution was treated with an equal volume of saturated ammonium sulfate solution, and filtered through folded filters to remove precipitated globulin and stromata. Saturated ammonium sulfate was then added until the hemoglobin separated in the cold. The product was filtered off, washed with ammonium sulfate solution, and dialyzed after the addition of a little ether. Finally, 1 per cent of sodium chloride was added.

Preparation B, a solution of oxyhemoglobin, was obtained according to the method recently published by one of us.[17]

The oxyhemoglobin was recrystallized three times as outlined in the method referred to, and washed several times with water saturated with carbon dioxide and oxygen. The crystals were ground to a thin paste with 0.85 per cent sodium chloride solution, diluted further with saline, dissolved with the minimum amount of normal sodium hydroxide solution, and diluted with saline to a total hemoglobin content of 8 to 10 per cent. The solution was first filtered through a Berkefeld V filter, then through a sterile Berkefeld N filter, and was preserved under sterile conditions.

Preparation C, also a solution of oxyhemoglobin, was prepared by following the method used for Preparation A as far as the addition of an equal volume of saturated ammonium sulfate solution.

It was found that if the ether used was repeatedly washed with water, and then dried first over calcium chloride and then over stick potassium or sodium hydroxide, the formation of methemoglobin was avoided. In fact, crystallization of the oxyhemoglobin occurred so rapidly after addition of the ammonium sulfate solution that it was rarely possible to filter the mixture without losing much of the pigment. The filtrate from the globulin and stromata was allowed to stand overnight in the ice box and the solution was then decanted from the heavy deposit of oxyhemoglobin crystals. These were filtered on a Buchner funnel and washed, and were recrystallized once and dissolved according to the methods used for Preparation B, omitting the preliminary filtration through a Berkefeld V filter.

8 to 10 per cent hemoglobin solutions prepared as above were used for injection. Seventeen rabbits in the three groups each received seven to eleven intraperitoneal injections of 8 to 15 cc. at weekly intervals. Only with a small proportion of the animals did we obtain strongly reacting sera, that is, sera which gave a heavy precipi-

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[17] Heidelberger, M., *J. Biol. Chem.*, 1922, liii, 31.

tate with 0.01 per cent hemoglobin solution within a few minutes. In the case of Preparation A one out of five rabbits gave a strongly reacting serum, and two gave weaker sera; with Preparation B, two out of five rabbits gave strongly reacting sera, while with Preparation C, in spite of the use of seven rabbits, only weak sera were obtained, that is sera which, under the conditions given below, gave readily perceptible, but relatively weak turbidities or slight precipitates.

Thus we have found, as did Demees[4] and Browning and Wilson,[7] that hemoglobin is a rather weak antigen, a fact which may account for the negative results of the experiments of others. Possibly, however, a greater formation of antibodies could be achieved through intravenous injection as was done by Hektoen and Schulhof. It is also not impossible that some of the impurities associated with hemoglobin in the cell may actually facilitate antibody formation by the hemoglobin. By the use of the especially sensitive ring test, as employed by Hektoen and Schulhof[16] and Higashi,[14] other sera would undoubtedly have been found positive.

The Specificity of Antihemoglobin Sera.

The tests were carried out as follows: 2 to 3 capillary drops (1 drop = 0.04 cc.) of the serum were added to 0.2 cc. of 0.01 per cent solutions of the different hemoglobins. In Table I is given the intensity of the turbidity or precipitate after the tubes were shaken and had stood for various periods at room temperature. Solutions of crystalline horse and dog oxyhemoglobin were used in the tests, while in the case of other animals, red cells were laked with water and ether, centrifuged, and the resulting solution was filtered through asbestos or kieselguhr paper (Macherey) and made up to a definite hemoglobin concentration. Serum 1 was obtained with Preparation A, the others, with Preparation B.

After the test mixture had stood overnight in the ice box, some further weakly positive reactions were noted. Precipitin reactions of equal intensity were obtained with methemoglobin, carbon monoxide hemoglobin, and cyanhemoglobin prepared from crystalline horse oxyhemoglobin.

The reactions in Table I show that the serological specificity of the antibodies produced by crystalline hemoglobin is no less developed than in the case of the serum proteins, which have been repeatedly and thoroughly studied from this standpoint (Nuttall[18]). Strong

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[18] Nuttall, G. H. F., Blood immunity and blood relationship, Cambridge, 1904.

TABLE

Specificity of Antihe 'obin Sera.

	Read after	Human.	Dog.	Horse.	Donkey.	Pig.	Ox.	Sheep	Goat.	Rabbit.	Guinea pig.	Rat.	Mouse.	Chicken.	Pigeon.
Serum 1	1 hour	0	0	+++	++±	0	0	0	0	0		0	+	0	0
" 2	5 min.	0	0	++±	++	0	0	0	0	0	0	0	0	0	
" 2	20 "	M.Tr.	0	+++	++±	0	0	Tr.	0	0	0	0	0	0	
" 2	1 hour	" "	0	+++	+++	0	M.Tr.	"	M.Tr.	0	0	0	M.Tr.	0	
" 3	20 min.	0	0	++±	++	0	0	0	0	0	0	0	0	0	
" 3	30 "	0	0	++±	++	0	0	0	0	0	0	0	0	0	
" 3	1 hour	0	0	++±	++	0	0	0	0	0	0	0	M.Tr.	0	
" 4	30 min.	0		+±	+		0						" "		
" 4	1 hour	0		++	+±		0						" "		

race; M.T trac

reactions were given with the homologous horse hemoglobin and a somewhat weaker precipitation with the blood pigment of the closely related donkey, while the other red cell solutions gave little or no reaction. This marked precipitation of the hemoglobin of a closely related species parallels the findings with serum proteins, and shows that with hemoglobin as well, the serological or chemical relationship runs parallel to the morphological. In agreement with this conclusion, already stated in the preliminary paper,[13] Higashi[14] has likewise found a definite species-specificity in precipitin, complement fixation, and anaphylactic reactions. An exception was found by him in the case of closely related species. Moreover, anti-chicken and anti-goose hemoglobin sera gave reactions of equal or nearly equal intensity with the hemoglobin of the pigeon and sparrow.

The serological findings are manifestly a proof that the crystallographic differences found by Reichert and Brown[19] in their comprehensive work are principally the result of chemical differences in the hemoglobin, a conclusion which has hitherto not been universally accepted,[20] since conditions of crystallization and impurities present undoubtedly do influence crystalline form.[21]

With respect to the serological differences between hemoglobin and other proteins, our immune sera, like those obtained by Demees and Higashi, had a very weak hemolytic action and either gave no reaction at all with horse serum albumin, or only a very faint trace on long standing. On the other hand, it was possible, by means of the inhibition reaction,[22] to trace a relationship between hemoglobins of different origin. The observed effect was less, however, than that recorded in our preliminary publication.

To each of a number of tubes was added 0.2 cc. of 0.01 per cent horse hemoglobin solution and 0.1 cc. of a solution of red cells of the animal indicated and containing approximately 0.1 per cent of hemoglobin. 0.1 cc. of 1 per cent of sodium chloride solution was added to a control tube and 0.08 cc. of Serum 3 was put in all. The results are shown in Table II.

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[19] Reichert, E. T., and Brown, A. P., *Carnegie Inst. Washington, Pub. No. 116*, 1909. *Cf.* Loeb, J., *Science*, 1917, xlv, 191.

[20] *Cf.* Robertson, T. B., Principles of biochemistry, Philadelphia and New York, 1920.

[21] For additional evidence of chemical differences see Landsteiner, K., and Heidelberger, M., *J. Gen. Physiol.*, 1923–24, vi, 131.

[22] (*a*) Camus, M. L., *Compt. rend. Acad.*, 1901, cxxxii, 215. (*b*) Landsteiner, K., and Halban, T., *Münch. med. Woch.*, 1902, xlix, 473. (*c*) Landsteiner, K., *Biochem. Z.*, 1918–19, xciii, 115; 1920, civ, 280.

It is not unlikely that the inhibition observed in these tests depends upon the existence in the various hemoglobins of a common component, such as hematin, in view of the analogous inhibition shown in the case of protiens containing a common radical which had been introduced into the molecule.[22,c] However, a solution of hematin itself caused no inhibition, just as it failed to give reactions with precipitin sera.[23]

TABLE II.

Inhibition of Precipitation.

Dog.	Horse.	Rabbit.	Chicken.	Control
Tr.	0	±	+	++

Identity of the Precipitable Substance with Hemoglobin.

Because of the negative results of many authors, the difficulty of producing strongly reactive antibodies, and the observations of Hektoen and Schulhof, it is of great importance to settle the question as to whether the antigen in our experiments is really hemoglobin itself, or some impurity difficult to remove.

The most striking result reported by Hektoen and Schulhof was that the precipitin reaction remained unaltered in solutions in which the hemoglobin had been destroyed with acetic acid and the globin removed. However, hydrochloric acid, when not greatly diluted, destroyed the antigen.

We repeated the experiments with acetic acid and hemoglobin at room temperature in the concentrations given by the authors, removing test portions from time to time. After removal of the globin by neutralization, a series of dilutions was made and compared with dilutions of the original hemoglobin solution. Precipitin tests were made on these solutions, and rough estimations were made of how much of the original antigenic value remained, by comparing the acid-treated tubes with the hemoglobin dilutions. In this way it was found that after ½, 2, and 22 hours, about 50, 60, and 75 per cent, respectively, of the antigen had been destroyed. Part of the hemoglobin was still spectroscopically demonstrable even after the action of 0.01 N hydrochloric acid instead of 0.1 N acetic acid for 24 hours.

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[23] Gay, F. P., quoted by Schmidt and Bennett.[11]

In view of this fact, the following experiment was set up.

A 10.2 per cent solution of twice recrystallized oxyhemoglobin was prepared by dissolving a suspension of the crystals with the aid of the minimum amount of normal sodium hydroxide. 50 cc. of the solution were diluted to 100 cc., and although neutral to litmus, the solution was acidified with 2 cc. of normal acetic acid in order to avoid any question as to its reaction. 50 cc. of the resulting 5 per cent solution were mixed with 50 cc. of 0.05 N hydrochloric acid and allowed to stand at room temperature for 4½ hours. The brown solution was then neutralized with 0.1 N ammonium hydroxide containing 5 per cent of ammonium chloride, allowed to stand overnight, and centrifuged. A determination of hemoglobin by Stadie's method[24] showed 0.2 of the original amount still to be present, while a similar solution, allowed to stand overnight before neutralization, still contained 0.1 of the original amount. The solutions showed the absorption bands of methemoglobin, and reacted with immune serum with approximately the degree of intensity to be expected from the analytical findings.

It thus becomes evident that, under the conditions given, hemoglobin is not completely split into hematin and globin.

The fact that the antigen is destroyed to an appreciable extent in a short time by acetic acid of a lower concentration than 0.05 N is in itself strong support for the belief that hemoglobin is the substance active in the serological tests; for other antigens such as the serum proteins are influenced in their precipitin reactions only by acid of much higher concentration.

To sum up, there are a number of grounds for ascribing the antigenic action to hemoglobin itself. In the first place, removal of the stromata by filtration and of globulins by ammonium sulfate followed by recrystallization, leaves the precipitability of the hemoglobin solution intact, as does also treatment with aluminium cream according to Marshall and Welker,[25] as Hektoen and Schulhof found. In the second place, the observation that our immune sera reacted to an equal extent with solutions of purified hemoglobin and with extracts of red cells containing all the accompanying impurities points in the same direction. In the third place, the precipitin reaction permits the detection of hemoglobin in as low a concentration as 0.001 per cent, which compares favorably with the strength of common precipitins. It would be difficult to reconcile this fact, and that of the inhibition found at a concentration of 0.05 per cent, with the assumption of an active impurity. Finally, it can be shown that hemoglobin is actually carried down in the precipitate formed in hemoglobin solutions by immune serum. The important observation that the precipitate is red was originally made by Leblanc and Ide.[26] Since it could be objected that the hemoglobin might have been carried down by adsorption, and not have been actually involved in the precipitin reaction, tests were made under conditions which eliminate this possibility.

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[24] Stadie, W. C., *J. Biol. Chem.*, 1920, xli, 237.

[25] Marshall, J., and Welker, W. H., *J. Am. Chem. Soc.*, 1913, xxxv, 820.

The following immune sera of rabbits were used.

A. Antihemoglobin Serum 2	20 drops
B. " " 3	20 "
C. Anti-horse serum	10 "
D. Anti-human "	20 "
E. Anti-donkey "	20 "

The tubes were set up as follows:

In each tube 0.2 cc. of 0.1 per cent hemoglobin solution plus
1. 1.8 cc. saline + A.
2. 1.8 " " + B.
3. 1.8 " 1:500 horse serum + C.
4. 1.8 " 1:500 human " + D.
5. 1.8 " 1:500 donkey " + E.

At the end of 1 hour the precipitates were centrifuged off, washed with saline, and again centrifuged. The differences observed were very striking. The sediment in Tubes 1 and 2 was definitely red, while that in the other tubes was pure white in spite of the fact that the antigen-antibody complex was precipitated in a hemoglobin solution of the same concentration as that in the first two tubes. One is forced to the conclusion that the red color of the precipitate in the hemoglobin-antihemoglobin system is actually due to antigen-antibody combination.

The following additional experiment was run.

1 cc. of 8 per cent hemoglobin solution was diluted with water to 30 cc. and treated with 20 cc. of 0.01 N hydrochloric acid. After 2 hours the globin and

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[26] See Leblanc,[2] p. 362; Ide,[3] p. 266.

hematin were precipitated by neutralization. The precipitin test showed in comparison with hemoglobin solutions of known strength that about 80 per cent of the antigen in the pale yellow-brown solution had been destroyed. 1 cc. of the solution was then treated with enough antihemoglobin serum to induce maximum precipitation, after which the precipitate was handled as in the preceding experiment. In this case, too, the sediment was definitely red, showing that the antibodv was bound to a pigmented antigen and not to a colorless split product.

From the above experiments, therefore, it would seem that in order to prove definitely that the antigen or an active part of it can be split from the hemoglobin, it would be necessary to show conclusively that any active solution obtained by treatment with acid contains no hemoglobin, methemoglobin, or closely related derivative.

CONCLUSIONS.

Sera produced by immunization with crystalline oxyhemoglobin react species-specifically with hemoglobin solutions.

Evidence is presented that in this reaction the hemoglobin itself is the active substance.

Conversion of oxyhemoglobin into methemoglobin, carbon-monoxy hemoglobin, or cyanhemoglobin does not alter the response to the precipitating immune serum.

Not only the hemoglobin of homologous species, but also that of other species causes inhibition in greater or less degree of the precipitin reaction.

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STUDIES ON THE ETIOLOGY OF SNUFFLES IN STOCK RABBITS.

PARANASAL SINUSITIS A FACTOR IN THE INTERPRETATION OF EXPERIMENTAL RESULTS.

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INTRODUCTION.

With the hope of throwing more light on the group of upper respiratory diseases in man, we have sought to study a similar affection in laboratory animals,—rabbit snuffles. In choosing this subject, it was apparent that there would be no lack of material because snuffles is almost ubiquitous among stock rabbits, occurring in endemic form practically all the time, and in epidemic form at periodic intervals. Furthermore, the study concerned an ailment, the incitant of which is still in doubt, although some affirm *Bacillus bronchisepticus*, and others *Bacillus lepisepticus*, as the etiological agent. In view of the prevalence of the disease and the frequency with which the rabbit is employed in experimental work, additional findings in this field should prove of importance to the laboratory worker.

Literature Regarding Etiological Agents.—*Bacillus lepisepticus* is regarded as the incitant of rabbit snuffles by Beck,[1] Kraus,[2] Roger and Weil,[3] Volk,[4] and others. *Bacillus lepisepticus*, first accurately described in 1887 by Theobald Smith,[5] is now classed with the Pasteurella or hemorrhagic septicemia group of microorganisms. An examination of the articles quoted shows that in no instance has uncomplicated rabbit snuffles, free from septicemia, been induced with this bacillus. Indeed, in the hands of some investigators, as, for example, Volk, this organism, when applied to the uninjured nasal mucous membranes of rabbits, fails to cause snuffles; applied, however, to the scarified mucosa, it induces death, apparently from septicemia, but without clinical signs of snuffles. Similarly, Roger and Weil's experiments demonstrate that this bacillus causes experimental rabbit septicemia which, only at times, is associated with what appears to be a symptomatic rhinitis.

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[1] Beck, M., *Z. Hyg.*, 1893, xv, 363.

[2] Kraus, R., *Z. Hyg.*, 1897, xxiv, 396.

[3] Roger, H., and Weil, E., *Arch. méd. exp. et anat. path.*, 1901, xiii, 459.

[4] Volk, R., *Centr. Bakt., 1te Abt., Orig.*, 1902, xxxi, 177.

[5] Smith, Theobald, *J. Comp. Med. and Surg.*, 1887, viii, 24.

On the other hand, an organism first described by Tartakowsky,[6] and rediscovered by Ferry in 1910[7] as the supposed cause of canine distemper, called by the latter *Bacillus bronchisepticus*, was considered later (1912) by Ferry[8] and by M'Gowan[9] and by Ferry and Hoskins[10] as the incitant of snuffles. In the words of Ferry and Hoskins: "It seems to be evident that the ordinary form of snuffles as encountered in the various rabbitries in this country, characterized by a variable nasal discharge accompanied by sneezing and rubbing the nose, with more or less loss of appetite and weight and a rather subacute or chronic course (which constitutes the large majority of cases), is caused by *B. bronchisepticus;* while the more acute form and the most fatal, in the majority of instances is due to *Bact. lepisepticum.*" These writers do not believe that snuffles is a single entity, and quote Ward[11] who could produce snuffles by injecting rabbits intravenously with either *Bacillus ozænæ*, *Bacillus proteus*, or *Bacillus bronchisepticus*.

A number of other microorganisms has been described by different investigators[12] as causing rabbit snuffles, but the literature fails to confirm their specific relationship.

It appears then from the work of previous writers that the question as to the actual causation of rabbit snuffles is still unanswered. Furthermore, there is no clear conception of the definition of the uncomplicated disease. One group of investigators who maintain that *Bacillus lepisepticus* is the etiological agent regards snuffles as a stage or symptom of septicemia.

The investigations reported in this paper have to do with snuffles as it occurs among laboratory stock rabbits. We shall first describe the disease encountered in such animals so as to present the basis on which our experiments were made.

[6] Tartakowsky, M.-G., *Arch. Sc. biol.*, 1898, vi, 255.

[7] Ferry, N. S., *Am. Vet. Rev.*, 1910, xxxvii, 499.

[8] Ferry, N. S., *Am. Vet. Rev.*, 1912–13, xli, 77; *J. Path. and Bact.*, 1913–14, xviii, 445.

[9] M'Gowan, J. P., *J. Path. and Bact.*, 1911, xv, 372.

[10] Ferry, N. S., and Hoskins, H. P., *J. Lab. and Clin. Med.*, 1919–20, v, 311.

[11] Ward, H. C., *J. Infect. Dis.*, 1916, xix, 153.

[12] Hutyra, F., and Marek, J., Spezielle Pathologie und Therapie der Haustiere, Jena, 4th edition, 1913, ii, 17.

Description of the Disease.

Snuffles as it occurs spontaneously in rabbits kept under laboratory conditions may be divided clinically into different groups: the intermittent and the chronic, which are the commonest types, and the acute, which is infrequent.

Intermittent snuffles is to be noted especially in rabbits which are observed over long periods—many months or even years. A typical attack begins with an acute nasal discharge of a slight amount of thin mucus, which may be transitory and can scarcely be called snuffles. In some cases, the process may go on to a mucopurulent or purulent stage with sneezing, rubbing the nose, soiling and consequent matting of the hair of the paws, loss of appetite and of weight. After a number of days, these symptoms subside and the animal returns to normal apparently. Under conditions which reduce the resistance, to be described later, as also spontaneously, several attacks may occur in the course of a year.

In the chronic form of snuffles, the rabbit has a persistent, white, tenacious, nasal discharge accompanied frequently with crust formation on the nostrils. There is to be noted sneezing when nasal obstruction is present, along with rubbing of the nose and soiling and matting of the hair of the paws which are frequently depilated in small areas. The disease may progress to a destruction of the nasal bones with a deformity simulating in outward appearance luetic destruction of the nose in man. Infrequently there forms a localized abscess about the nasal fossæ. The animal may become adapted to this chronic condition so that there is little, if any, loss of weight, or emaciation.

The acute variety is not common, to judge from our experience with stock rabbits of 800 to 3,000 gm. or more in weight, and a few months to several years of age. Indeed, we believe that this condition can best be seen in breeding establishments where animals are observed from the first weeks of life. When it does occur among laboratory stock it is a manifestation of some underlying disease, such as septicemia. In these cases there is a thin, glairy discharge from the nose, and a rapid weakening of the animal leading to death. From the nasal discharge, blood, and organs, especially the lungs, *Bacillus lepisepticus* can be isolated. Or the acute attack may accompany pneumonia or other infections and resemble the nasal disorder occurring in a variety of primary conditions such as hay-fever, common colds, influenza, the exanthemata, and seen after exposure to irritants or cold, in man. The failure to distinguish acute, uncomplicated snuffles from the nasal disorder secondary to some underlying disease has led some previous investigators into error when they ascribed to the incitant of snuffles, the etiological agent of the disease responsible for the false "snuffles" they observed.

Sinusitis in Snuffles.

In early experiments we noted the frequency of paranasal sinusitis in apparently normal animals and in those suffering from snuffles. This condition interfered with our procedures to such an extent that an extensive study was begun to determine its nature.

Anatomy of the Rabbit's Nose.—The frequency with which the rabbit suffers from nasal affections is probably due to the complexity of the nasal passages.

The turbinate bones which contain numerous folds are situated on the posterior and lateral walls. The maxilloturbinate, a finely ridged mass of bone, occupies the anterior portion of the lateral wall, while the ethmoturbinate, which consists of much broader folds, is placed posteriorly. Each of these folds or serrations is covered with nasal epithelium so that numerous pockets are present. Moreover, the nasolachrimal ducts discharge into the nasal passages. Besides these structures there are various paranasal sinuses. Congestion of the mucous membrane with resultant swelling may convert the folds or sinuses into closed cavities, thus permitting infectious processes or purulent material to remain localized. The tendency for closed pockets of infection to develop has, we believe, a considerable bearing on the production of the clinical condition known as snuffles and on the interpretation of the results of experimental inoculation.

Occurrence of Sinusitis.—A systematic examination was made of the upper air passages of thirty-four rabbits, among which were animals with snuffles. Included in this number were carefully selected normal rabbits which had been under observation for several months.

The examination included a thorough investigation of all the organs including the brain. The nasal passages were exposed by cutting away the bones of the nose and frontal region of the skull. In order to avoid irritation of the mucous membrane by general anesthetics, such as chloroform or ether, the rabbits were killed by quickly severing the upper cervical spinal cord.

Of the thirty-four rabbits, twenty-three were found to be suffering from intermittent or chronic snuffles. Of these latter, twenty-two had a chronic inflammation of the mucous membrane of the upper nasal passages associated with a purulent paranasal sinusitis.

In the cases of long duration, all the sinuses were filled with thick, white, tenacious, inspissated, odorless pus; while in those of shorter duration only one or two sinuses were so affected. The mucous membrane lining the turbinates and the sinuses was thickened but showed no signs of congestion or acute catarrhal inflammation. In some instances, the thickening of the mucosa resulted in a pocket, the opening of which revealed the firm pus already described. On microscopic examination, the epithelial cells were seen to be necrotic and exfoliated in areas. The subepithelial zone was thickened by an extensive exudation of polymorphonuclear and endothelial leucocytes, or monocytes, but without congestion. Some edema was also present, and in Giemsa-stained preparations, bacteria were noted in the ciliary layer and in the subepithelial zone, mainly phagocytosed. Giemsa's, Gram's, and methylene blue stains of the pus showed numbers of mononuclear cells and somewhat less abundant, polymorphonuclear elements. Many of these cells were disintegrated like those in the pus found, as a rule, in chronic infectious processes. That is, the cell outlines were blurred, the nuclei indistinct, and the cell granules few in number. Numerous broken down cells with granules liberated and scattered were seen. Few bacteria, but these often of different species, were found in phagocytosis as well as outside the cells. The culture findings will be described further on.

Of the thirty-four rabbits, two showed signs of slight snuffles, that is, there was no actual nasal discharge, but merely a dampness about the nostrils, and some sneezing, but none of the other signs of a typical attack, as, for example, soiling or matting of the hair of the paws. Such cases are usually designated in the literature as "beginning snuffles." In both instances was found a chronic inflammatory process of the mucosa and sinuses similar to that met with in chronic snuffles.

Of the original series, nine rabbits were carefully selected as having no ailment whatever, and certainly no obvious signs of nasal affection. These animals were under observation for several months, and were regarded as normal stock. Two of the nine showed the chronic inflammatory process of the mucosa of the nasal passages associated with the purulent sinusitis above described, which is similar in its pathological picture to that exhibited by the animals with typical chronic snuffles.

In another series of 121 rabbits[13] which were carefully selected, individually caged, and kept under a rigid quarantine, five showed moist noses, or what might be considered as slight snuffles. In these five, chronic sinusitis was found. Among the remainder, carefully examined for any possible indication of past or present disease, and determined by this rigid test to be outwardly normal, nine revealed a chonic inflammatory condition of one or more of the nasal sinuses.

Bacteriological Examination.—As Ferry and Hoskins pointed out[10] there is no one organism constantly associated with the condition known as snuffles, although they believe *Bacillus bronchisepticus* causes the chronic, and *Bacillus lepisepticus* the acute disease.

In the present study, the material constituting the nasal discharge was obtained during life by means of a swab, and at autopsy the pus from the sinuses and nasal passages was secured. The nasal discharge and pus were stained and examined microscopically for bacteria and cellular elements. Cultures were made by streaking the pus on rabbit blood agar plates which were incubated for 24 to 48, or more hours, at 37°C.

It may be stated that no one organism was found constantly associated with the condition. The commonest microorganisms isolated were, in the order of their frequency, *Staphylococcus albus*, *Bacillus bronchisepticus*, *Bacillus lepisepticus*, *Micrococcus catarrhalis*, a hemolytic mucoid, Gram-negative bacillus, a diphtheroid, and other unidentified bacteria occasionally. Stained film preparations of the nasal discharge and pus from the upper passages showed, as a rule, an extensive degeneration of cells and a limited phagocytosis of microorganisms, features indicating, as already described, that the exudates were chronic in nature.

When more than one sinus is involved, different bacteria may be present in the various sinuses, as the following protocol illustrates.

Rabbit SA, a stock animal, suffering from snuffles of unknown origin and duration, was killed by pithing. The orbital sinus contained thin, glairy pus which yielded a pure culture of a hemolytic mucoid organism resembling those of the Friedländer group of bacilli. The paranasal sinus was filled with thick, white, tenacious, inspissated pus from which a pure culture of *Bacillus bronchisepticus* was obtained. The anterior nares, on the other hand, contained thick, white pus which on culture showed a mixture of both of the microorganisms mentioned.

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[13] We are deeply indebted to Doctors Brown and Pearce of The Rockefeller Institute for supplying us with a number of these animals and with other helpful assistance.

In this instance, illustrative of other findings, the chronic sinusitis in different regions was associated with two distinct bacteria.

In later observations on more than 50 rabbits the incidence of chronic sinusitis was found to be still greater than in those first studied, both as concerns apparently normal animals and those showing chronic, intermittent, or beginning snuffles.

To summarize, it appears that practically 100 per cent of stock rabbits suffering from chronic, intermittent, or beginning snuffles reveal old, chronic, inflammatory processes of the upper nasal passages, while 9 per cent of apparently normal stock animals, free from any symptoms whatever, exhibit similar chronic purulent conditions of the upper nasal passages. Ordinarily, on casual selection of supposedly normal rabbits, this proportion is still higher and may reach 25 per cent of the total. The bacteria most commonly met with are *Staphylococcus albus*, *Bacillus bronchisepticus*, and *Bacillus lepisepticus*. In other words, in about one-tenth of a carefully selected group of normal stock rabbits and in as many as one-fourth of a casually selected group, chronic infectious processes involving one or more of these microorganisms can be found in the nasal passages, *before any experimental procedure is begun.*

Transmission Experiments.

The facts already presented show the difficulties of interpretation of experimental procedures to induce snuffles. It is desirable, before stating our attempts at transmission, to direct attention to certain other factors which may favor the spontaneous onset of the disease, or symptoms simulating the affection. Dust or mould in samples of defective hay, employed for feeding purposes, may give rise to acute irritation of the nasal passages associated with a certain amount of mucoid discharge and also with conjunctivitis. This condition appears to be due purely to mechanical irritation and not to infection. The disease itself may be induced in apparently normal animals by lowering the resistance, as by subjecting them to low temperatures. An analogy can be found in the experiments of Mudd and his coworkers [14] in which it is demonstrated that chilling the body surface of man may induce "colds." Another factor in reducing the resistance of rabbits is the injection of killed vaccines.[15] In view of the presence of chronic disease in the nasal passages of rabbits supposedly normal, it is presumed that lowering the resistance of the animals causes an extension or increase of the previously existing inflammatory process, thus converting what may be termed a potential snuffles into an active and intermittent type of the disease.

The importance ascribed to *Bacillus bronchisepticus* and *Bacillus lepisepticus* as the etiological agents of rabbit snuffles suggested to us an attempt to induce the disease *de novo* with these cultures, with due regard for the factors which might interfere with the experimental results.

Experiments with Bacillus lepisepticus Cultures.—A pure culture of *Bacillus lepisepticus* [16] was employed in the following experiment. It was originally obtained from snuffles secretion and its virulence had been enhanced by intrapleural passage through twelve consecutive rabbits.

Thirteen young rabbits (about 800 to 1,000 gm. in weight) were used. These were selected because such are said to be especially susceptible to this microorganism[17] and also to exclude, if possible, previous attacks of snuffles. The material inoculated consisted of a heavy suspension (about 5,000 million per cc.) of an 18 hour growth on blood agar seeded directly from the pleural exudate of the twelfth rabbit. 0.5 cc. of the bacterial suspension was forcibly injected into each nostril by means of a 1 cc. syringe.

Only three of the rabbits showed symptoms of snuffles which appeared on the 6th, 13th, and 13th day, respectively, after inoculation. On these days the rabbits were killed. The autopsy of the first animal revealed thick, white, inspissated pus in the left nasal sinus, and the mucous membrane of the anterior nares was slightly injected. In stained film preparations of the pus, mononuclear cells predominated over the polymorphonuclears. Most of the cells were degenerated: they were poorly and irregularly stained, the nuclei were indistinct and the granules few in number. There were a few small bacilli present, mostly in various stages of phagocytosis. Cultures yielded hemolytic, mucoid, Gram-negative bacilli in pure state but no *Bacillus lepisepticus*. In the second rabbit there was marked coccidiosis of the liver. A small pocket of inspissated pus was noted in the right nasoturbinal region, but no congestion of the mucosa. Cultures of the pus showed *Bacillus lepisepticus*. The third animal had only a slight mucopurulent discharge from the nose without involvement of the sinuses.

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[14] Mudd, S., Grant, S. B., and Goldman, A., *Ann. Otol., Rhinol. and Laryngol.*, 1921, xxx, 1.

[15] For example, of two rabbits injected intravenously with killed cultures of *Bacillus bronchisepticus* and of four injected subcutaneously with vaccines of *Bacillus lepisepticus*, all showed typical snuffles after two to six inoculations at a time when the serum contained agglutinins against these microorganisms in titers of 1:150 to 1:500.

[16] This culture was obtained through the kindness of Doctor L. T. Webster.

[17] De Kruif, P. H., *Proc. Soc. Exp. Biol. and Med.*, 1921–22, xix, 34.

Ten of the animals of this series remained free from snuffles from 8 to 10 days after inoculation. They were all killed at the end of the period of observation to determine the presence of sinusitis. Six were normal, but in the other 4, chronic inflammatory conditions were found in the mucous membranes of the upper nasal passages, together with inspissated pus in the orbital and nasal sinuses of one, and in the nasal sinus alone in the remaining three. From the pus of one of these three, no growth was obtained; from that of the second, both *Bacillus bronchisepticus* and *Staphylococcus albus* were isolated, and from that of the third, a hemolytic, mucoid, Gram-negative bacillus was cultured. The rabbit in which both orbital and nasal sinuses were affected showed in the pus of the orbital sinus *Bacillus bronchisepticus*, and in that of the nasal sinus *Bacillus lepisepticus*.

From the foregoing experiments, it will be noted that of thirteen animals inoculated intranasally with a heavy dose of a culture of *Bacillus lepisepticus*, highly pathogenic for rabbits, only three exhibited snuffles. In two of these three a sinusitis was found, the pathological picture of which revealed an inflammatory condition of long standing, probably antedating the inoculations. From the pus of one of the sinuses, *Bacillus lepisepticus* was recovered, but from that of the other, a different bacillus was isolated. The third rabbit showed no chronic inflammation of the upper nasal passages but a considerable time, 13 days, had elapsed between the injection of *Bacillus lepisepticus* and the onset of symptoms. Ten rabbits remained free from snuffles, but in four of these chronic sinusitis was found, associated with various bacteria. An animal showing two distinct sinus infections had different organisms in the sinuses.

We conclude therefrom that *Bacillus lepisepticus* cannot, by itself, be considered as the incitant of snuffles, for, if a group of supposedly normal stock rabbits be observed over the same length of time employed in our experiments, findings like those in an inoculated group can be obtained even without manipulation, as we have demonstrated.

We now attempted transmission experiments with *Bacillus bronchisepticus*.

Experiments with Bacillus bronchisepticus Cultures.—Our experience with this microorganism closely resembled those with *Bacillus lepisepticus*.

Of six young rabbits inoculated intranasally, in the manner already described, with a culture of *Bacillus bronchisepticus* in its second generation or first subplant from the growth obtained from snuffles material, only one animal exhibited the disease. This took the form of a slight mucopurulent discharge from the nose, which appeared 10 days after inoculation and lasted for 1 day, after which the rabbit returned to normal. The animals were observed over a period of 5 weeks.

As with *Bacillus lepisepticus*, these results indicate that *Bacillus bronchisepticus* cannot be considered as having a specific relationship to the initiation of snuffles in supposedly normal stock rabbits. It is possible, in view of the evidence already presented, that the induction of transitory snuffles in one instance is apparently due to an acute exacerbation of a chronic inflammatory process in the nasal passages, present prior to the experimental procedure.

Attempts to induce snuffles by the inoculation of these supposed etiological agents having failed, experiments were now made in which the nasal discharge itself, or suspensions of the nasal mucosa from typical cases of the disease in stock rabbits were employed. It was thought that perhaps some non-cultivable agent in these secretions, or the conjoint action of *Bacillus bronchisepticus*, *Bacillus lepisepticus*, and *Staphylococcus albus* which were present in them, could operate to induce the disease.

Experiments with Unfiltered Nasal Secretions.—Two series of experiments were made.

In one series, the nasal secretions from recent and old cases of snuffles were collected by means of a thin cotton nasal swab and then this material was introduced into the nasal cavities of young, supposedly normal stock rabbits.

In another series, the snuffles animal was killed by pithing, or a blow on the head, and the entire length of the nasal mucous membrane was dissected out. This was then ground with sand and 10 cc. of saline solution, and 0.5 cc. of the supernatant fluid injected into each nostril by means of a syringe.

Altogether, material was employed from twelve rabbits with early snuffles; that is, animals killed after the onset of the first definite symptoms of the disease. It was inoculated into twenty-two young rabbits.

Of the twenty-two animals, seven showed clinical snuffles. Of these, six were killed and all showed a marked chronic inflammatory process in the nasal passages with suppuration in one or more sinuses. Table I summarizes the results in the seven positive cases.

In this experiment, as in the preceding ones, no definite evidence of the transmissibility of the disease by means of the nasal secretions from snuffles could be obtained. For in six of the seven positive cases, were found old, chronic affections in the nasal passages. From the pathological findings of thickened mucous membrane and inspissated pus in the sinuses, containing cells in varying stages of degeneration with relatively few bacteria, mostly phagocytosed, we infer that this nasal condition antedated the inoculation. Furthermore, the irregularity in the period of incubation and the inconstant presence of any one or any single group of microorganisms, together with the preexisting chronic disease of the nasal passages in supposedly normal controls, all indicate that the inoculations were not specifically related to the appearance of snuffles. The evidence supports our earlier observations

TABLE I.

Rabbit No.	Time after inoculation snuffles appeared.	Sinus infection.		Cultures.
		Right.	Left.	
	days			
1*	10	0	0	0
2	3	+	0	*Bacillus bronchisepticus*, *Bacillus lepisepticus*, and *Streptococcus viridans*.
3	1	+	+	*Bacillus bronchisepticus*.
4	1	0	+	" *lepisepticus* and coliform organism.
5	3	+	+	*Staphylococcus albus;* few *Bacillus bronchisepticus*.
6	4	+	+	*Bacillus bronchisepticus;* few *Staphylococcus albus*.
7	10	+	+	" *bronchisepticus*, *Bacillus lepisepticus*, and *Staphylococcus albus*.

* Rabbit 1 showed snuffles 10 days after inoculation. The symptoms consisted of a scanty mucopurulent discharge which disappeared in 3 days. The animal was not killed.

that the inoculations incite a latent condition to become an active disease.

Although the unfiltered material failed to give conclusive results, we now used filtered suspensions of the nasal mucous membranes from snuffles animals and inoculated them both intranasally and intracerebrally in young stock rabbits.

Two stock rabbits with typical snuffles were killed by pithing, and the nasal and sinus mucous membranes were dissected out. The resulting material was ground with sand and 10 cc. of saline solution, centrifuged lightly to remove large particles, and the supernatant fluid was passed through a Berkefeld V candle. The clear filtrate showed no growth on the usual media.

The filtrate, 0.5 cc. into each nostril, was inoculated into each of three young rabbits, but these failed to show any symptoms.

The experiment was repeated three times, employing different sources for the snuffles material, but in all cases the results were negative or indecisive.

Five different samples of filtrates were inoculated intracerebrally in doses of 0.25 to 0.35 cc. into eight young rabbits. In no instance did there develop clinical evidence of snuffles, nor any untoward symptoms.

Thus both the unfiltered and the filtered suspensions of snuffles material, the unfiltered inoculated intranasally, the filtered, intranasally and intracerebrally, failed to induce the disease in supposedly normal stock rabbits.

DISCUSSION AND SUMMARY.

In our experience, covering a large number of rabbits, we have found that the condition known as snuffles falls into different types, the acute and fatal, symptomatic of some underlying infection such as septicemia or pneumonia; and the intermittent, and the chronic. The intermittent and the chronic types considered in this paper are those most commonly present in laboratory stocks.

Our observations point to a widespread prevalence of the disease among rabbits kept under laboratory conditions. This statement might be questioned had the stock we examined been derived from a single source. But the animals were procured from dealers who obtain rabbits in New York, Pennsylvania, Ohio, and as far west as Michigan. The same dealers supply the stock of most of the laboratories in the East. Hence we believe that this disease is found generally prevalent in this part of the country.

We have demonstrated that practically all rabbits with intermittent or chronic snuffles reveal old chronic inflammatory processes of the upper nasal passages associated with thick inspissated pus in one or more sinuses. Moreover, these conditions are also present in about one-tenth of carefully selected, supposedly normal stock rabbits and in one-fourth of a casually selected group, free, during long periods of observation, from any of the symptoms of active snuffles. The peculiar anatomy of the animal's nose which predisposes to the ready formation of enclosed pockets of purulent material may be the cause for the chronicity of upper nasal affections. When the animal's resistance is lowered, the long standing, inflammatory process can flare up into an acute exacerbation, and then show itself as typical snuffles. Various means can effect this: chilling the body, the intravenous injection of foreign proteins such as killed vaccines, or the intranasal inoculation of microorganisms of divers species. Bacteriological examination of the nasal secretions or sinus pus from animals with snuffles and those apparently free of the disease shows the presence in both cases of various microorganisms—*Staphylococcus albus*, *Bacillus bronchisepticus*, *Bacillus lepisepticus*, and others, in order of frequency. Different bacteria may be found in different sinuses in the same animal.

A lack of recognition of these factors has led, we believe, to erroneous conclusions with regard to the inciting agent of the disease. *Bacillus lepisepticus* and *Bacillus bronchisepticus* have been declared the incitants of snuffles. Our experiments, in which an attempt was made to induce the disease *de novo* with these microorganisms, failed. In all cases (with a single exception) in which snuffles followed, there was evidence of an infection which, judging from the condition of the nasal passages and from the cells in the exudates or secretions, had existed before the inoculations were made. Furthermore, the microorganisms recovered from the nasal passages had as a rule no relationship to those in the material inoculated. We attempted also to produce snuffles by inoculating intranasally the unfiltered and filtered suspensions of the ground nasal mucous membranes from typical cases of the disease occurring in stock rabbits. These attempts also failed.

It appears, therefore, that intermittent and chronic snuffles, as it attacks rabbits kept under laboratory conditions, is, as a rule, a sign of an underlying condition—an exacerbation of a chronic inflammatory process in the upper nasal passages, associated with a purulent paranasal sinusitis. The microorganisms recovered are to be looked upon as tending to maintain such conditions but we have still been unable to reproduce typical snuffles with them, employing supposedly normal stock rabbits for the purpose. One may presume that some agent, as yet undetermined, diminishes the resistance of the nasal mucosa, allowing different bacteria to invade and multiply there, thus causing disturbance. In this respect perhaps an analogous condition exists to that which prevails in epidemic influenza and common colds in man.

It is obvious that further work along these lines cannot be properly carried out with rabbits whose antecedent history is unknown. The problem of the incitant of snuffles can best be studied in a breeding stock which is well controlled, one affording an opportunity to observe the animals from an early period of life.

CONCLUSIONS.

A study of snuffles in stock rabbits reveals that this disease is associated with a chronic inflammatory condition of the upper nasal passages together with purulent paranasal sinusitis. The outward signs of snuffles are, as a rule, the expression of an exacerbation of preexisting inflammation in the upper nasal mucosa. While it is conceivable that in early or incipient cases the disease may be uncomplicated and independent of chronic inflammatory processes, we have not been able to observe such cases. Supposedly normal stock rabbits, even those carefully selected and quarantined, exhibit the chronic inflammation just referred to. Such animals cannot therefore be employed for the solution of the problem of the actual incitant, which is, as yet, undetermined.

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IMMUNITY STUDIES OF ROCKY MOUNTAIN SPOTTED FEVER.

II. Prophylactic Inoculation in Animals.

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Of the many urgent and interesting problems regarding Rocky Mountain spotted fever, that of personal prophylaxis seems to be the most important. The experiments reported here were carried out in an attempt to devise a practical method by which a non-immune population might be rendered immune to the bites of infected ticks.

Ricketts and Gomez[1] early recognized the possibility of developing a satisfactory method of preventive inoculation, and carried out a few experiments in this connection but no adequate quantitative experimental analysis of the factors concerned in the production of artificial immunity. Since we have no pure culture of the etiological agent and cannot recognize it microscopically, we must rely solely upon animal inoculations for quantitative as well as qualitative estimation of the presence of the spotted fever organism. Since the number of causative organisms must bear a definite relationship to the amount of antibodies or the degree of immunity which is produced, a knowledge of the number and virulence of the organisms used is a prerequisite to an understanding of the phenomena of immunization. For example, 1 cc. of the citrated blood of guinea pigs with all the symptoms of spotted fever may contain only 1 or as many as 1,000 minimum lethal doses, but this fact can be ascertained only by careful titration of the material by animal inoculations, and unless the number of minimum lethal doses is determined in each instance, the immunization experiments carried out become meaningless or even misleading. Moreover, since the virus dies out in citrated or defibrinated blood or serum, even at 4°C., it must be titrated simultaneously with the vaccination or subsequent immunity tests in which it is being used.

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[1] Ricketts, H. T., and Gomez, L., Studies on immunity in Rocky Mountain spotted fever, *J. Infect. Dis.*, 1908, v, 221.

The selection of experimental animals is another important factor, and since our aim is to develop methods of protection for the human being, we must choose an animal in which the morbidity and mortality with respect to spotted fever infection approach as closely as possible those of man. The guinea pig excels other animals in this respect. The rabbit is far less susceptible than the guinea pig, and monkeys (*Macacus rhesus*) are extremely susceptible, those of a series of eighteen having shown 100 per cent morbidity and mortality. The age and size of the animal are very important. Full grown males, weighing 500 to 600 gm., are to be recommended; first, because of their comparatively greater resistance to banal laboratory infections and their undiminished and remarkably constant susceptibility to the spotted fever infection, and second, because the characteristic genital lesions are best observed in male animals. The consideration of such factors as just outlined is absolutely essential for formulating a practicable method of prophylactic vaccination.

The ordinary immunological procedures, known to be effective in other diseases, have been subjected to experimental determination of their possible application in the case of spotted fever: (1) the effect of injection of non-infective quantities of live spotted fever virus; (2) the effect of injection of killed virus; (3) the effect of injection of dead virus; (4) the effect of injection of freshly prepared neutral or superneutral mixtures of the live virus with immune serum; (5) the effect of heating and of age upon the immunizing properties of neutral mixtures of the virus with immune serum.

The two strains of spotted fever virus used in the present series of experiments were obtained through the kind cooperation of Dr. R. R. Parker, and we continue to use his designation of them as "Cooper" and "Barlow." The guinea pigs were almost always adult males weighing 500 to 600 gm. The virus was used in the form of citrated blood (equal parts of blood and citrate solution), usually drawn from the heart of the infected guinea pig on the 4th to 6th day of fever, preferably when the scrotal lesions became noticeable. Blood drawn on the 2nd or 3rd day of fever contains, in my experience, far less of the virus. Every specimen of blood was tested for bacterial contamination on ordinary broth and slant agar and was titrated simultaneously with each experiment to determine the number of killing doses which it contained. All the injections of virus, or mixtures containing virus, were made intraperitoneally. The temperatures of the animals were taken every morning during the period of observation; that is, until death or recovery, or, in case no infection developed, for at least 2 weeks. The immune serums used in this work were chiefly from rabbits which had passed through a typical but non-fatal infection and had been bled 2 to 3 weeks after the temperature returned to normal.[2] Scrotal lesions were frequently observed in the infected rabbits. In some instances the immune serums came from rabbits which had received a second dose of virus (4 cc. citrated blood of the guinea pig) during convalescence; these serums are designated as "reinforced," and their titers are somewhat higher than those of the convalescent serums. All the immune serums were kept in the refrigerator at 4°C. except at time of use.

TABLE I.

Effect of Non-Infectious Quantities of Virus.

Date of inoculation.	Material.	Amount.	Result.	Immunity test.
1922		*cc.*		
May 10.	Citrated guinea pig blood, Cooper strain (1 M.L.D. = 0.01 cc.).	0.001	No reaction.	June 13, 1922 (33 days), Cooper strain 1 cc. (1,000 M.L.D.); typical infection but recovered.
		0.0001	" "	Similarly tested; typical infection; death in 11 days.
		0.00001	" "	Similarly tested; typical infection; death in 11 days.
Jan. 25.	Citrated guinea pig blood, Barlow strain (1 M.L.D. = 1 cc.).	0.1	" "	Feb. 17, 1922 (23 days), Barlow virus 0.5 cc. (5 M.L.D.); typical infection; death in 10 days.
		0.01	" "	Similarly tested; typical infection; death in 19 days.

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[2] Noguchi, H., Immunity studies of Rocky Mountain spotted fever. I. Usefulness of immune serum in suppressing an impending infection, *J. Exp. Med.*, 1923, xxxvii, 383.

TABLE II.

Effect of Injection of Killed Virus.

Date of inoculation, December 8, 1922.

Material.	Killed by.	Amount.	Result of inoculation.	Result of immunity test, 28 days later, against Barlow virus 0.1 cc. (1 M. L. D.).
		cc.		
Citrated guinea pig blood (Barlow + Cooper strain),* 100 M.L.D. in 1 cc.	Chloroform.	1	No reaction.	Typical febrile reaction; recovery.
		0.3	" "	Severe infection with typical scrotal lesions; recovery.
Same.	Ether.	1	" "	Severe infection with typical lesions; recovery.
		0.3	" "	Typical infection; died in 9 days.
		0.1	" "	Severe infection; necrotic skin and ear lesions; recovery.
"	Xylene.	1	Slight fever.	Severe infecton with necrotic lesions of scrotum and ear lobes; recovery.
		0.3	No reaction.	Typical severe infection; recovery.
		0.1	Slight fever.	Severe infection; died in 19 days.
"	Heating to 56°C. for 10 minutes.	0.75	No reaction.	Typical severe infection; recovery.
		0.3	" "	Typical infection; died in 14 days.
		0.1	Irregular fever (secondary infection?).	Typical infection.
Same virus, 1 cc. + immune serum, 1 cc.		1	No reaction.	No infection.
Same virus, 1 cc. + immune serum, 1 cc.		0.3	" "	Slight fever; no symptoms of spotted fever.
Same virus, 1 cc. + immune serum, 1 cc.		0.1	" "	Mild fever; no lesions; recovery.

Titration of virus used for testing immunity. January 5, 1923.

Barlow virus (guinea pig citrated blood).	Result of inoculation.
cc.	
1	Typical infection; killed for virus in 5 days.
0.1	" " died in 14 days.
0.01	Suspicious fever; recovery.
0.001	" " "

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* Kept in refrigerator for 20 hours.

Effect of Non-Infective Quantities of Live Spotted Fever Virus.

That guinea pigs which survive a non-fatal but typical spotted fever infection resist subsequent infection has long been known, but whether or not animals receiving too small a quantity of the virus to become infected would acquire increased resistance remained to be determined. Ricketts and Gomez published some observations on this point,[1] stating that, although they had made no systematic attempt to determine the vaccinating properties of minute doses of virus, they had reason to believe that prophylactic vaccination could not be based on this principle. The present experiment shows (Table I) that no appreciable immunity develops under these conditions. Three guinea pigs received non-infective doses of one strain and two of another, and all succumbed to subsequent inoculation 33 and 23 days later, respectively.

Effect of Injection of Virus Killed Either by Chemicals or by Heating.

Chloroform, ether, and xylene were allowed to act upon the virus by shaking with it for 5 minutes. After the mixtures had been in the refrigerator (4°C.) for 20 hours, the clear portion in each case was used for inoculation. Guinea pigs were given the sterilized virus in doses of 1, 0.3, and 0.1 cc., and the animals were tested for immunity 28 days later by inoculation of a single minimum lethal dose of Barlow strain (0.1 cc.). There was no evidence of any increased resistance to the infection.

Another portion of the same virus was heated to 56°C. for 10 minutes in a water bath. Three guinea pigs were then inoculated with 0.75, 0.3, and 0.1 cc., respectively. No infection followed, and 28 days later each animal was given one minimum lethal dose of the virus (Barlow strain, 0.1 cc.). All had severe and typical infections, one dying in 14 days (Table II).

The failure of the animals to derive any immunity from the introduction of the killed virus was apparently due to modification of the immunizing substances by the chemical or physical agents, since the same quantities of virus (100, 30, and 10 M.L.D.) when mixed with immune rabbit serum, proved ample, as will be shown later, to render animals immune to the inoculation of the same test material.

Effect of Injection of Dead Virus.

Although the foregoing experiment had shown that the virus could not be killed without impairment of its immunizing properties, the question remained whether it might not retain its power to confer immunity if allowed to die out under such unfavorable conditions as, for example, low temperature for a long period. Hence several animals, which had been inoculated with specimens of virus kept in the refrigerator (4°C.) for periods of 28 to 69 days and had had no infection, were tested 40 days later for immunity by the injection of 100 M.L.D. of virus (Barlow strain, 1 cc.). The animals all developed typical and, in most instances, fatal infections (Table III. Compare also Table IX, under the heading "Controls (heated virus alone).").

TABLE III.

Effect of Injection of Dead Virus.

Strain.	Kept at 4°C. for a period of.	Amount.	Result of inoculation.	Result of immunity test, 40 days after inoculation, against Barlow virus 1 cc. (100 M. L. D.).
	days	*cc.*		
Cooper Guinea pig 11	69	2	No reaction; died of secondary infection in 9 days.	
Same	69	4 (of 1:6 solution in citrate).	No reaction.	Typical infection; died in 16 days.
Cooper Guinea pig 19	57	2	" "	Typical infection; died in 12 days.
Cooper Guinea pig 30	31	2	Typical infection; killed for material.	
Cooper Guinea pig 31	28	2	No reaction.	Typical infection; finally recovered.
Cooper Guinea pig 47	21	1	" "	Typical infection; died in 15 days.
Barlow Guinea pig 24	38	2	" "	Typical infection; died in 12 days.

TABLE IV.

Effect of Injection of Neutral or Subneutral Mixtures of Virus and Immune Serum.

May 10, 1922. Virus: citrated guinea pig blood (Cooper strain), 1 cc. (100 M.L.D.).
Immune serum: rabbit.

Immune serum No.	Amount.	Vaccination reaction.	Immunity test.	
			Interval after vaccination.	Degree of protection.
	cc.			
11	0.1	Mild fever; recovery.	33 days.	Complete against 1,000 M.L.D.
			10 mos.	“ “ 30 “
	0.01	“ “ “	33 days.	“ “ 1,000 “
23	0.1	“ “ “	33 “	“ “ 1,000 “
	0.01	Typical infection; recovery.	33 “	“ “ 1,000 “
			8 mos.	“ “ 1 “
			9 “	“ “ 5 “
25	0.1	Mild fever; recovery.	33 days.	“ “ 1,000 “
	0.01	Moderate fever; recovery.		
186	0.1	Mild fever; recovery.	10 mos.	“ “ 30 “
	0.01	Moderate fever; recovery.		
307	0.1	Mild fever; recovery.	10 mos.	“ “ 30 “
	0.01	Typical infection; recovery.		
314	0.1	Mild fever; recovery.	3 mos.	“ “ 1,000 “
	0.01	Moderate fever; recovery.	3 “	“ “ 1,000 “
316	0.1	Mild fever; recovery.	8 “	“ “ 1 “
	0.01	Moderate fever; recovery.		
326	0.1	Mild fever; recovery.	6 mos.	“ “ 100 “
	0.01	Typical infection; recovery.		
327 (reinforced).	0.1	No reaction.	9 mos.	Complete against 5 M.L.D.
	0.01	Moderately severe infection.		

330 (reinforced).	0.1	No reaction.	33 days. 10 mos.	Complete against 1,000 M.L.D. " " 30 "
	0.01	Mild fever; died of intercurrent infection.		
341 (reinforced).	0.1	No reaction.	33 days 10 mos.	" " 1,000 " " " 30 "
	0.01	Moderate fever; recovery.		
350 (reinforced).	0.1	No reaction.	33 days. 10 mos.	" " 1,000 " " " 30 "
	0.01	Moderate fever; recovery.		

Titration of virus used for testing immunity. June 13, 1922.

Cooper strain virus.	Result of inoculation.
cc.	
0.1	Typical infection; death in 9 days.
0.01	" " " " 13 "

Effect of Injection of Freshly Prepared Neutral or Superneutral Mixtures of Live Virus with Immune Serum.

It had been noticed early in these studies of immunity in spotted fever that guinea pigs which had received suitable mixtures of virus and immune serum remained well throughout several weeks of observation. The mixtures were innocuous, irrespective of the amount of virus introduced, provided the amount of immune serum were correspondingly adjusted. For example, 1 cc. of virus containing 100 or 1,000 M.L.D. might be injected without causing any infection when mixed with 0.1 cc. or more of immune serum, and animals so inoculated were found to be completely refractory to subsequent inoculation with as many as 100 or 1,000 M.L.D. The degree of immunity acquired was altogether comparable with that of animals which had received a subneutral mixture and had passed through a mild course of fever or a moderately severe infection with scrotal lesions. The immunity produced by subneutral mixtures is apparently the same as that following convalescence in animals which survive a typical severe infection caused by virus alone.

Table IV shows the neutralizing power of the serum of eight convalescent rabbits and of four hyperimmunized immune rabbits (*i.e.* which had received a second injection of virus during convalescence) in 0.1 and 0.01 cc. against 100 M.L.D. (Cooper strain, 1 cc.) and the degree of immunity developed in the guinea pigs. 0.1 cc. of the convalescent serum was an almost, but not completely, neutralizing dose, while the same quantity of the reinforced immune serums was sufficient to neutralize all infectivity. The guinea pigs were tested for immunity on several different occasions and with virus of various degrees of virulence. Of the eight animals tested 33 days later with 1,000 M.L.D., none became infected, and no change in temperature was observed. Two tested 3 months afterwards against 1,000 M.L.D. resisted infection. One animal was tested after 6 months with 100 M.L.D. and proved resistant. Several others were tested after longer periods—8, 9, and 10 months—against 1, 5, and 30 M.L.D. of virus and likewise proved immune.

Superneutral Mixtures.—In the case of human vaccination the neutral mixtures would leave too narrow a margin between the neutral and the subneutral, and no margin can be too great to insure safety for the persons vaccinated. Since, however, a great excess of the immune serum in a mixture might reduce or even nullify the immunizing properties of the virus, it was necessary to determine this point. Hence a series of experiments was carried out in which a considerable excess of the serum was assured by using 1 cc. against 100 M.L.D. of virus. Fourteen different samples of immune serum, twelve of which been used also in the previous series of experiments, were used (Table V), all but one of which (No. 11) completely neutralized the virus. The animals were tested for immunity after 1, 4, 7, and 7½ months, respectively, and found to be completely

TABLE V.

Effect of Injection of Superneutral Mixtures of Virus and Immune Serum.

May 25, 1922. Virus: citrated guinea pig blood (mixed Cooper and Barlow strains), 1 cc. (100 M.L.D.). Immune serum· rabbit.

Immune serum No.	Amount.	Vaccination reaction.	Immunity test.	
			Interval after vaccination.	Degree of protection.
	cc.			
11	1	Mild fever; recovery.	7 mos.	Complete against 1 M.L.D.
23	1	No reaction.	7 "	" " 1 "
25	1	" "	18 days.	Died of intercurrent infection; no lesions of spotted fever after injection of 1,000 M.L.D. virus.
186	1	" "	1 mo.	Complete against 100 M.L.D.
			4 mos.	" " 1,000 "
307	1	" "	1 mo.	" " 100 "
314	1	" "	4 mos.	" " 1,000 "
316	1	" "	4 "	" " 1,000 "
326	1	" "	4 "	" " 1,000 "
327 329	1	" "	Reserved for later test.	
330	1	" "	4 mos.	Complete against 1,000 M.L.D.
			7 "	" " 1 "
			7½ "	" " 5 "
332	1	" "	7½ "	" " 5 "
349	1	" "	7½ "	" " 5 "
350	1	" "	Reserved for later test.	

TABLE VI.

Effect of Various Quantities of Immune Serum on the Immunizing Power of the Virus.

June 24, 1922. Virus: citrated guinea pig blood (Barlow strain), 1 cc. (100 M.L.D. or 1,000 M.I.D.).
Immune serum: rabbit.

Immune serum No.	Amount.	Vaccination reaction.	Immunity test.	
			Interval after vaccination.	Degree of protection.
	cc.		*mos.*	
23	2	No reaction.	5½	Complete against 100 M.L.D.
			6	" " 1 "
			6½	" " 5 "
	2	" "	3	" " 1,000 "
	1	" "	3	" " 1,000 "
			7	" " 1 "
			7½	" " 5 "
	1	" "	3	" " 1,000 "
			7	" " 1 "
			7½	" " 5 "
	0.5	" "	7	" " 1 "
			7½	" " 5 "
	0.5	" "	Lost by intercurrent infection after 19 days.	
	0.25	Slight fever; recovery.	Reserved for later test.	
	0.25	Definite febrile reaction; recovery.	3	Complete against 1,000 M.L.D.
	0.1	Mild fever; recovery.	3	" " 1,000 "
	0.1	Severe fever; no scrotal lesions; recovery.	Reserved for later test.	
25	2	No reaction.	3	Complete against 1,000 M.L.D.
	2	" "	Reserved for later test.	
	1	" "	3	Complete against 1,000 M.L.D.
	1	" "	5½	" " 100 "

	0.25	Some irregular rises of temperature, no spotted fever symptoms; recovery.	7½	Complete against	5 M.L.D.
	0.25	No spotted fever symptoms.	Died of intercurrent infection in 23 days.		
	0.1	Mild fever; recovery.	7	Complete against	1 M.L.D.
	0.1	" " "	Reserved for later test.		

Titration of mixed virus (Cooper-Barlow) used for testing immunity. June 24, 1922.

Citrated guinea pig blood.	Results of inoculations
cc.	
1	Typical severe infection with very marked scrotal lesions; died in 9 days.
0.1	Typical severe infection with very marked scrotal lesions; died in 10 days.
0.01	Typical severe infection with very marked scrotal lesions; died in 16 days.
0.001	Severe infection with extensive necrosis of scrotal skin, soles, and ear lobes; recovered.
0.0001	No infection.

resistant to infection with virus containing in some instances as many as 1,000 minimum lethal doses.

The results showed that the immune serum may be added to the virus in great excess without unfavorably influencing the development of immunity.

TABLE VII.

Duration of Passive Immunity.

June 24, 1922.

Immune serum (rabbit) No.	Amount.	Result.	Immunity test.
	cc.		
23	1	No reaction.	Tested 12 days later against Cooper virus 1 cc.;* typical infection; died in 9 days.
25	1	" "	Tested 12 days later against Cooper virus, 1 cc.;* mild infection; recovery. Tested 2 months later against Cooper virus, 1 cc. (1,000 M.L.D.); no infection.
	1	" "	Tested 2 months later against Cooper virus, 1 cc. (1,000 M.L.D.); typical severe infection; died in 11 days.

Titration of Cooper strain virus used for immunity tests. September 25, 1922.

Guinea pig citrate blood.	Result of inoculation.
cc.	
0.5	Severe infection with scrotal lesions; killed for virus in 10 days.
0.1	Severe infection with scrotal lesions; killed for virus in 10 days.
0.01	Severe infection; died in 13 days.
0.001	" " " " 13 "
0.0001	No infection.

* Control died in 14 days.

To confirm and supplement the above experiments, another series of animals was inoculated with mixtures containing the same amount of virus with varying amounts of immune serum. Two immune serums were separately used, and for each dose two animals were inoculated. 2, 1, 0.5, 0.25, and 0.1 cc. of serum, respectively, were mixed with 1 cc. of virus containing 100 minimum lethal doses or about 1,000 minimum infecting doses. No reaction followed the inoculation in the animals which received the mixture containing 0.5 cc. or more of serum, but animals which received 0.25 cc. or less all had some degree of febrile reaction, although no scrotal lesions were noted. Irrespective of whether the quantity of serum was subneutral, neutral, or superneutral, all the animals proved to be equally resistant to several subsequent attempts to infect them after intervals of 3 months (with 1,000 M.L.D. of virus), 5, 5½, 6, and 7 months (with 1 to 5 M.L.D. of virus) (Table VI). It is evident, therefore, that the quantity of immune serum may be liberally increased if the mixture is to be used for vaccination.

TABLE VIII.

Protective Power of the Blood of Vaccinated Guinea Pig.

Date of vaccination, May 25, 1922.

Material: virus (Cooper and Barlow strains mixed), 1 cc. + immune rabbit serum, 1 cc.

This animal belongs to the series reported in Table V.

Interval after vaccination.	Test of blood for protective power of citrate plasma.*	Result of inoculation.
days		
30	1 cc. + virus, 0.1 cc. (10 M.L.D. or 100 M.I.D.)	Definite febrile reaction; recovered.
	0.2 cc. + same virus, 0.1 cc.	Typical severe infection, with scrotal and ear lesions; recovered.
	0.1 " + " " 0.1 "	Typical severe infection; died in 10 days.

* 10 cc. heart blood were mixed with 10 cc. citrate solution; plasma collected after centrifugation.

Of the fourteen other guinea pigs vaccinated on the same day with the same material, two were tested after the same interval (30 days) by injection of virus; both resisted infection with 100 M.L.D. of the same virus as used in the above experiment. The remaining twelve animals were tested at intervals of 4 to 7½ months with 1 to 1,000 M.L.D. of virus, and all resisted infection (see Table V).

The question arose whether the immunity which developed after the inoculations of neutral or superneutral mixtures might not be merely a passive immunity, due to the introduction of the immune serum. This point was decided by the experiment recorded in Table VII which showed that the inoculation of 1 cc. of a powerful immune serum does not protect a guinea pig from subsequent infection 12 days or later; hence the immunity observed in guinea pigs 2 weeks or longer after the inoculation of neutral or superneutral mixtures cannot be ascribed to a passive immunity brought about by the antiserum introduced with the virus.

Protective Power of the Blood of Vaccinated Guinea Pigs.

From the theoretical point of view it was interesting to determine the neutralizing power of the blood of a guinea pig which had previously been inoculated with a neutral mixture of virus (Cooper-Barlow strains, 1 cc.) and immune serum (No. 28, 1 cc.). Blood was withdrawn from the heart 30 days after the time of vaccination. The citrated plasma was separated, and 1, 0.2, and 0.1 cc. were tested against 10 M.L.D. of the virus (Cooper strain, 0.1 cc.). The largest quantity, 1 cc., failed to neutralize the virus completely, but the animal recovered, while 0.2 and 0.1 cc. had no protective effect (Table VIII).

Fourteen other guinea pigs, vaccinated at the same time, and tested after the same period by injection of 100 M.L.D. of virus, all resisted the attempt to infect them. Hence the vaccinated guinea pigs are able to neutralize a far greater quantity of virus *in vivo* than the low neutralizing titer of their blood would lead us to expect.

Effect of Heating and of Age upon the Immunizing Properties of Neutral Mixtures of Virus and Immune Serum.

The strikingly effective protective value of a neutral or superneutral mixture of virus and immune serum against experimental spotted fever in guinea pigs suggested the possibility of applying the principle to human beings. The prospect of sero-vaccination, under the most rigid quantitative supervision of the virus and immune serum, is promising; nevertheless, it seems important, for the time being at least, to devise some method in which the virus in the mixture is killed, but which still gives a vaccine of definite protective value.

In Tables IX and X are recorded the results of experiments in which guinea pigs were inoculated with neutral or superneutral mixtures of virus and immune serum which had either been heated to 60°C. for

20 minutes in a water bath or preserved in the refrigerator (4°C.) for 32 days. One set of animals was inoculated with portions of the

TABLE IX.

Effect of Heating upon the Neutral Mixtures of Virus and Immune Serum.

October 5, 1922. Virus: citrated guinea pig blood, Cooper and Barlow strains (1 cc. contained 100 M.L.D.), 1 part. Immune serum: mixture of serum from several immune rabbits, 1 part.

Amount	Result of vaccination.	Immunity test.		
		Interval after vaccination.	Tested against.	Degree of protection.
		Unheated.		
cc.				
2	No reaction.	18 days.	10,000 M.L.D.	Complete.
2	" "	18 "	10,000 "	"
2	" "	2 mos.	100 "	Died of secondary infection.
Controls (normal rabbit serum + virus).				
2	Typical infection; died in 11 days.			
2	Typical infection; died in 11 days.			
		Heated to 60° C. for 20 minutes.		
2	No reaction.	18 days.	10,000 M.L.D.	Mild fever, recovery.
2	" "	18 "	10,000 "	" " "
2	" "	2 mos.	100 "	Died of secondary infection.
		Controls (heated virus alone).		
2	No reaction.	18 days.	10,000 M.L.D.	Typical infection; died in 15 days.
1	" "	18 "	10,000 "	Typical infection; died in 12 days.
1	" "	2 mos.	100 "	Typical infection; died in 13 days.

freshly prepared mixture; another set received a portion of the same mixture, which had been previously heated; the remainder of the mixture was placed in the refrigerator and inoculated 32 days later.

Suitable controls (virus mixed with normal rabbit serum, and virus alone) accompanied each of the three sets of experiments.

TABLE X.

Effect of Age upon the Neutral Mixtures of Virus and Immune Serum.

Portions of the mixtures of virus and immune serum used on Oct. 5, 1922 (Table IX) were kept in the refrigerator at 4°C. until Nov. 6, 1922 (32 days).

Amount.	Result of vaccination.	Immunity test.		
		Interval after vaccination.	Tested against.	Degree of protection.
cc.				
2	No reaction.	16 days.	100 M.L.D.	Typical infection; died in 15 days.
2	" "	1 mo.	100 "	Irregular fever (secondary infection).
1	" "	16 days.	100 "	Suspicious febrile reaction but no lesions; died in 22 days of secondary infection.
1	" "	5 mos.	30 "	No infection.
Controls (normal rabbit serum + virus).				
2	Secondary infection.			
1	No reaction.	16 days.	100 M.L.D.	Typical infection; died in 13 days.
Controls (virus alone).				
2	No reaction.	1 mo.	100 M.L.D.	Typical infection; died in 19 days.
2	" "	16 days.	100 "	Typical infection; died in 14 days.
2	Suspicious febrile reaction; died in 16 days; spleen enlarged.			
1	No reaction.	1 mo.	100 "	Typical infection; died in 15 days.
1	" "	16 days.	100 "	Typical infection; died in 10 days.

Examination of the results shows, first that the mixture was superneutral, since there was no evidence of infection in any of the animals inoculated with the unheated fresh mixture. Two of these animals were tested 18 days later against 10,000 M.L.D. of virus, the other 2 months later against 100 M.L.D., and both resisted infection. The latter animal died of intercurrent infection 3 weeks after the date of the immunity test. Normal rabbit serum had, of course, no neutralizing effect on the virus, and the two guinea pigs receiving the mixture of normal serum and virus died after 11 days.

Of the three animals which received the heated mixture of virus and immune serum, two, which were tested 18 days later against 10,000 M.L.D. of virus, had a mild febrile reaction but showed no lesions; the other, which was tested 2 months later against 100 M.L.D., died of intercurrent infection after 12 days; there were no specific pathological findings, and if the animal had a spotted fever infection, it must have been very mild. There is a great contrast between these results and those in the guinea pigs inoculated with the heated mixture of virus and normal rabbit serum, all of which died of typical infection.

Of the animals inoculated with the aged mixture, two died when tested 16 days later; one had a typical spotted fever infection, the other probably succumbed to secondary infection while recovering from mild spotted fever. The third, tested 1 month later, had irregular fever, but no symptoms of spotted fever. Of the animals which received the aged mixture of virus and normal rabbit serum, one died in 13 days of typical infection, the other was lost by intercurrent infection before the time of test. The aged virus alone produced neither infection nor immunity in the four animals receiving it; the two tested 16 days later died in 10 and 14 days, respectively, and the two tested after 1 month died in 19 and 15 days.

The contrast between the effect of fresh unheated and heated mixtures is again strikingly brought out in Table XI, in which are presented the results of the inoculation of smaller quantities of the superneutral mixture of virus and immune serum; one series of animals receiving respectively 1, 0.1, 0.01, and 0.001 cc. of the unheated, another the same quantities of the heated mixture. The injection of the heated mixture definitely modified the course of a subsequent test infection, although complete immunity was not induced even in a dose as large as 1 cc.; the unheated mixture conferred protection in a dose of 0.1 cc., and even in the animal receiving 0.01 cc. the infection was not fatal.

These findings confirm the earlier experiments establishing the effectiveness of fresh unheated neutral mixtures of virus and immune serum in producing immunity in guinea pigs. The uselessness of heated or aged virus, alone, or with normal rabbit serum, is definitely determined. The presence of a definite immunizing property in the neutral mixture which had been heated to 56–60°C., and also, though in less degree, in the neutral mixture kept at 4°C. for 32 days, indicates the probable effectiveness of mixtures treated in this way for prophylactic vaccination in man, notwithstanding that these preparations fall much below the fresh mixtures in immunizing titer.

TABLE XI.

Effect of Superneutral Mixtures of Virus and Immune Serum in Various Amounts.

Virus: guinea pig serum, 1 cc., representing 1,000 M.L.D.

Immune serum: rabbit, 1 cc., representing ten times the quantity necessary to neutralize 1,000 M.L.D.

Amount.		Result of vaccination.	Result of immunity test, 34 days later, against 10 M. L. D. of virus.
		Unheated.	
cc.			
1	virus 500 M.L.D. serum 0.5 cc.	No reaction.	No infection.
0.1	virus 50 M.L.D. serum 0.05 cc.	" "	" "
0.01	virus 5 M.L.D. serum 0.005 cc.	" "	Severe infection; recovery.
0.001	virus 0.5 M.L.D. serum 0.0005 cc.	Definite mild febrile reaction.	No infection.
		Heated.	
1		No reaction.	Mild infection; no lesions; recovery.
0.1		" "	Moderately severe infection; recovery.
0.01		" "	Severe infection; recovery.
0.001		" "	Typical infection; died after 14 days.

GENERAL CONSIDERATIONS.

From the findings outlined, it appears that the immunizing properties of the Rocky Mountain spotted fever virus are almost, if not totally, removed by heating to 60°C. for 20 minutes or by prolonged preservation at a low temperature. On the other hand, the immunizing power remains fully preserved when the virus is mixed with immune serum in neutral or superneutral proportions, notwithstanding the fact that such mixtures are completely devoid of infecting power. Moreover, heating of such mixtures to 56–60°C. for 20 minutes, or prolonged preservation at refrigerator temperature, does not altogether destroy, though it markedly reduces, the immunizing power. Since normal rabbit serum does not in any way influence or retard the rapid disappearance of immunizing properties from the virus under similar conditions (heating or aging), the persistence of immunizing power in the neutral or superneutral mixtures appears to be due to a specific reaction which takes place between the virus (antigen) and the immune serum (antibody).

For the purpose of inducing artificial immunity in man, the heated neutral mixtures of spotted fever virus and a highly potent immune serum may be used. The serum of infected guinea pigs is preferable to that of rabbits as the source of the virus, a high degree of virulence being more readily and uniformly maintained in the guinea pig. Immune serum from hyperimmunized rabbits is readily prepared and is capable of attaining a high degree of potency.[2] These two factors, highly virulent strains of the virus, and a powerful immune serum, are essential to the production of an effective sero-vaccine. The superneutral mixture is prepared as follows:

Blood is drawn from infected guinea pigs at the height of fever (4th to 5th day) and the clear serum separated from the clot.[3] The titer should be such that 1 cc. contains 100 M.L.D. of virus. By pooling a number of serums (after first making sterility tests of the individual serums to insure absence of secondary infection), virus of uniform

[3] It is imperative to determine by culture methods that the blood is free from any secondary bacterial infection, especially that caused by members of the paratyphoid group of bacilli, which induce febrile and splenic reactions strikingly similar to the symptoms shown by guinea pigs on the 4th and 5th days of spotted fever infection.

virulence can be obtained in large quantity. Immune rabbit serum, of a titer such that 0.1 cc. will neutralize 100 M.L.D., is mixed with the virus in ten times the neutralizing dose. A less potent immune serum is not suitable, because an inconveniently large quantity would be required. The mixture is heated to 56°C. for 20 minutes in a water bath and preserved in the refrigerator until used. Two or more injections of 1 cc. of the mixture may be given subcutaneously.

The procedure just outlined is a preliminary one only. The ultimate object of these experiments is a method in which the active (unheated) sero-vaccine may be safely applied to human vaccination.

SUMMARY.

Freshly prepared mixtures of spotted fever virus and immune rabbit serum in neutral or superneutral proportions confer complete immunity on guinea pigs.

The mixtures undergo a considerable loss in immunizing power when heated to 60°C. for 20 minutes, but are still capable, if used in sufficient quantity, of conferring a degree of immunity on the vaccinated animal such that a subsequent experimental infection is rendered less severe and non-fatal.

Unheated mixtures which had been preserved in the refrigerator at 4°C. for a period of 32 days still retained a certain degree of immunizing property.

The virus alone, or mixed with normal rabbit serum, when allowed to die out by prolonged preservation at refrigerator temperature, or when killed either by heating at 60°C. for 20 minutes or by chemicals (chloroform, ether, xylene) does not induce immunity in guinea pigs.

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THE INFLUENCE OF THE FACTORS OF SEX, AGE, AND METHOD OF INOCULATION UPON THE COURSE OF EXPERIMENTAL SYPHILIS IN THE RABBIT.*

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INTRODUCTION.

Our knowledge of the factors which govern the course of syphilitic infection in the rabbit is still incomplete. Until a systematic study has been made of those likely to influence the reaction of this experimental animal to infection with *Treponema pallidum*, our conceptions of the mechanism by which the infection is controlled are likely to be fragmentary at best. This communication deals with a study of some of the factors which seemed worthy of investigation from the standpoint of their influence upon the course of the syphilitic infection. The particular ones studied were those of age, sex, and method of inoculation.

Stated in more precise terms, the object was threefold: (1) to contrast the character of the disease in a group of females inoculated intradermally with that observed in a group of males inoculated in the same manner; (2) to contrast the character of the disease in a group of males inoculated intradermally with that in a group of males inoculated by the intratesticular route; and (3) to contrast the reaction of a group of young males following intratesticular inoculation with that of a group of older males. Opportunity was also afforded for a comparison of the course of the disease in males in which the primary focus following intratesticular injection had been removed by castration, with the course in animals in which no such attempt at interference with the ordinary progress of the disease had been made. In addition, it was possible to observe the course of the infection in animals in which attempts were made to produce chancres by implantation within the scrotum of bits of syphilitic rabbits' testicles. However, the influence of the factors of route of inoculation, age, and sex were the principal ones selected for study.

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The experiment involved no new method of inoculation for the study of syphilis in the rabbit. All of the routes chosen have been used in the past, but there are not, to our knowledge, any published reports on a large series of animals inoculated simultaneously in various ways with the same material. So far as we are aware, the three factors studied in this experiment, age, sex, and method of inoculation, have not in the past been similarly studied on a given series of rabbits with identical infecting material. Experience with experimental syphilis in the rabbit has shown conclusively that a given series of animals inoculated at one time cannot be compared directly with a series inoculated at some other time, even when one is working with the same strain of *Treponema pallidum*. In any comparison of behavior of groups of animals toward infection with *Treponema pallidum*, it is essential that the various groups be inoculated at the same time, as well as with equal amounts of the same virus. Failure to observe this elementary rule will yield results which are of little value from a comparative standpoint in the study of experimental syphilis in the rabbit, hence the point is stressed here.

Technique.

The strain of *Treponema pallidum* used in these experiments was the Nichols strain isolated originally in 1912 from the spinal fluid of a case of neurorecidive (1). This strain, now nearly 11 years old, has had many passages through rabbits, and has attained a degree of virulence for that animal of such extent that it will produce primary lesions in 100 per cent, and under favorable conditions, will produce generalized lesions in from 80 to 100 per cent of animals inoculated intratesticularly, although the incidence of such lesions is at times very low. The immediate source of the material was testicular tissue of two rabbits that showed clinical evidence of orchitis 23 days after inoculation. The testicles were excised under ether anesthesia at a period when the syphilitic orchitis was progressing rapidly, portions of the body of the organ were finely minced with scissors, ground up in a sterile mortar with sterile salt solution, and the more fluid portion of the resulting emulsion, which contained numerous actively motile organisms by dark-field examination, used for injection. Other parts of the body of the testicle were cut into small bits, 2 to 3 mm. in thickness, and these pieces served for scrotal implantation.

Intradermal Inoculation.—This method was accomplished by inserting the needle in the subcutaneous tissue of the sheath of the penis or vulva, at a distance of about 1 cm. from the mucocutaneous border, directing the needle toward the latter and at the same time bringing the top of the needle (bevel up) toward the surface until it lay in the skin just beneath the superficial layer, where it could be easily seen, and carrying it as far as the mucocutaneous border.

Intratesticular Inoculation.—This method needs no description.

Scrotal Implantation.—This method has already been described (2) and need not be discussed at length.

In the case of the intradermal and intratesticular inoculations the same amount of emulsion was used throughout; namely, 0.1 cc. For obvious reasons, the scrotal implants could not be of exactly the same size, but were approximately so.

The animals belonged to a variety of breeds and were kept under observation for a period of 90 days. The selection of this period was based upon the course of events following a unilateral testicular inoculation, since experience with the strain had shown that within this interval the infection would pass through a more or less orderly evolution of primary and secondary lesions, while observation over longer periods would entail the possibility of confusion through the occurrence of relapses. Hence, this period was selected as the most suitable for the comparisons that we had in mind. No attempt was made to demonstrate treponemata in the lesions as they appeared because of the possibility of the trauma exerting an influence on the course of the infection.

In all, thirty-seven rabbits were inoculated, of which number two died from pulmonary disease during the course of the experiment and are excluded from consideration. The remaining thirty-five which are to be reported may be divided into six groups as follows: (A) five adult females (over 12 months) inoculated intradermally; (B) seven adult males (over 12 months) inoculated intradermally; (C) five young males (below 5 months) inoculated intratesticularly; (D) seven adult males inoculated intratesticularly; (E) five adult males inoculated intratesticularly, castrated; (F) six adult males inoculated by scrotal implantation. The inoculations were all made on October 5, 1922.

EXPERIMENTAL.

Unless expressly stated otherwise in the ensuing protocols it will be understood that all primary lesions developed at the site of inoculation.

Group A.

This group comprised five adult females. Each animal received 0.1 cc. of testicular emulsion intradermally at the mucocutaneous border of the vulva.

Rabbit No. 1.—New Zealand red. No lesions of any sort except slight inguinal and popliteal lymphadenopathy.

Rabbit No. 2.—Brownish grey. After 11 days an infiltrated area, 3.0 mm. in diameter, and inguinal adenopathy. Maximum diameter, 4.0 mm., attained on the 15th day, later disappearance, followed by two recurrences on the 40th day.

Rabbit No. 3.—Brown. Infiltrated area on the 10th day, 1.0 mm. in diameter, disappearing on the 12th day. Slight inguinal lymphadenopathy. No recurrence.

Rabbit No. 4.—Brown. Infiltrated area appeared on the 19th day, increased to 1 cm. in diameter on the 52nd day, and ulceration occurred, then the lesion gradually receded. Inguinal and popliteal lymphadenopathy.

Rabbit No. 5.—Black. Indurated area, 3.0 mm. in diameter on the 10th day, reached maximum diameter of 8.0 mm. on the 21st day, then diminished gradually. Inguinal and popliteal lymphadenopathy.

Recapitulation.—Of the five animals in this group, one animal showed no local lesion save the initial reaction following injection, while another showed a small infiltrated area which disappeared within 10 days. In a third animal the local lesion consisted at the most of a papule, 4 mm. in diameter, and in a fourth, the lesion consisted of a papule attaining a maximum diameter of 8 mm. In only one of the group did the lesion develop into a characteristic chancre with necrosis of the epithelium, and ulceration. Not one of the animals showed the presence of generalized lesions (bone, skin, eye) within the period of 90 days following injection. In this group, then, the disease in general manifested itself as an inconstant and relatively mild local reaction without clinical evidence of generalization, except for a comparatively slight and rather uncertain reaction in the inguinal and popliteal lymph nodes.

Because of the absence of any generalized lesions in this group it seemed worth while to determine, by transfer of lymph node material to normal rabbits' testicles, if the organisms had invaded the body in spite of the slight local reaction at the inoculation site. The popliteal lymph nodes were removed from each animal in the group and, after emulsification in salt solution, were injected into the testicles of two normal rabbits, respectively. Of the ten animals to which node transfers were thus made, all developed an orchitis containing treponemata within a period of not more than 62 days after transfer, demonstrating that in all of the five females, infection and general dissemination had taken place in spite of the relatively slight degree or total absence of lesions at the site of inoculation, and in spite of the failure of production of clinically recognizable generalized lesions.[1]

Group B.

This group consisted of seven adult males. Each animal received 0.1 cc. of testicular emulsion intradermally in the sheath of the penis. The individual protocols follow.

Rabbit No. 6.—Brownish grey. Infiltrated area, 1 mm. in diameter, 31st day; disappeared on the 42nd day. Transient and immediate inguinal lymphadenopathy. No popliteal lymphadenopathy.

Rabbit No. 7.—Brown. Papule, 2.0 mm. in diameter, on the 31st day; on the 61st day three papules appeared and increased in size during the period of observation. Right-sided orchitis on the 89th day. Inguinal adenitis developed early.

Rabbit No. 8.—New Zealand red. Infiltrated area, 2.0 mm. in diameter, appeared on the 10th day, increased in size, developed into a chancre with a maximum diameter of 8.0 mm. on the 74th day. Inguinal and popliteal lymphadenopathy.

Rabbit No. 9.—Albino. Infiltrated area appeared on the 6th day, increased in size, and became a typical chancre with maximum diameter of 1.5 cm. on the 74th day. Left-sided periorchitis on the 85th day. Inguinal and popliteal lymphadenopathy.

Rabbit No. 10.—Brown. Infiltrated area appeared on the 10th day, became a typical chancre with a maximum diameter of 1.8 cm., and was increasing in size at the end of the experiment. Skin papule on right foot on the 84th day, bilateral orchitis at same time. Skin lesion on the left foot and lesions of the left tarsus, the left fifth metatarsal, and the left ulna on the 89th day.

Rabbit No. 11.—Brown. Infiltrated area appeared on the 10th day, regressed, then became a chancre with a maximum diameter of 10 mm. on the 61st day. Inguinal and popliteal lymphadenopathy.

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[1] This portion of the work was carried out in the Syphilis Division of the Department of Medicine of the Johns Hopkins Medical School, and was participated in by Dr. Jarold E. Kemp, to whom thanks are due.

Rabbit No. 12.—Grey. Infiltrated area appeared on the 10th day, a maximum diameter of 5.0 mm. attained on the 19th day; the lesion then receded. No ulceration. Transient inguinal lymphadenopathy.

Recapitulation.—In this group of seven animals there was one in which the only local lesion was a transient tiny area of infiltration, a second in which the local lesion developed into an indurated area, 0.5 cm. in diameter at its maximum, and a third in which the local lesion consisted of three infiltrated papules. In this latter animal evidence of generalization of the virus was provided by an orchitis appearing on the 89th day following injection. In the remaining four animals characteristic chancres appeared on the prepuce at the site of inoculation, and in two of these animals generalized lesions occurred. Summarizing these results: one animal showed only a slight and transient local lesion; two showed persistent lesions which were in the nature of papules which did not ulcerate; and four developed typical chancres. Generalized lesions, consisting of metastatic orchitis, skin papules, or periosteal thickenings, developed in three of the animals within a period of 90 days; the lesions of skin or bones, however, occurred in only one animal of the group. In this group the primary reaction at the site of inoculation was much greater than in the group of females, and the regional lymphadenitis was more marked. Exclusive of testicular involvement, one of the seven rabbits showed generalized lesions, while none of the females showed such lesions.

Group C.

This group consisted of five young males, all less than 6 months of age. Each received 0.1 cc. of testicular emulsion containing spirochetes, in the right testicle. The individual protocols follow.

Rabbit No. 13.—Brown. Orchitis (right) appeared on the 10th day, scrotal edema on the 24th day, metastatic orchitis (left) on the 46th day, and a scrotal chancre (right) on the 60th day. Skin lesion appeared on the right foot on the 66th day.

Rabbit No. 14.—Brown. Nodular orchitis (right) appeared on the 17th day, scrotal edema on the 24th day, and metastatic orchitis (left) on the 40th day. Skin lesion appeared on the right foot on the 62nd day, and a similar lesion on the left foot on the 73rd day.

Rabbit No. 15.—Brown. Orchitis (right) appeared on the 10th day, a scrotal edema on the 19th day, metastatic orchitis (left) on the 46th day, and a scrotal chancre (right) on the 60th day. Skin lesion appeared on the left foot on the 60th day.

Rabbit No. 16.—Brownish grey. Orchitis (right) appeared on the 10th day; the entire testicle involved on the 24th day, after which the orchitis regressed, and no other lesions appeared.

Rabbit No. 17.—Greyish brown. Orchitis (right) appeared on the 17th day, scrotal edema on the 25th day, and metastatic orchitis (left) on the 48th day. Scrotal chancre (right) appeared on the 52nd day and skin lesions appeared on the right foot on the 70th day. A second scrotal chancre appeared on the 77th day on the right side.

Recapitulation.—Of the five young males injected intratesticularly an initial orchitis occurred in all. In four, or 80 per cent, an orchitis developed in the non-inoculated testicle within an average period of 43.7 days. All of these four animals showed skin lesions within an average period of 64 days. One animal only failed to develop metastatic lesions in the testicle or elsewhere.

The outstanding feature of the condition presented by this group of animals was the occurrence of severe primary lesions and of well developed lesions in the uninoculated testicle, as contrasted with comparatively slight lesions elsewhere, all of which were confined to the skin.

Group D.

This group comprised seven adult males inoculated intratesticularly with 0.1 cc. of testicular emulsion containing treponemata. Only one testicle was inoculated and no attempt was made to interfere with the course of the infection. The individual protocols follow.

Rabbit No. 18.—Himalaya cross. Orchitis (right) appeared on the 10th day, a scrotal chancre (right) on the 42nd day, and metastatic periorchitis on the 46th day. Skin papule appeared on the right foot on the 62nd day.

Rabbit No. 19.—Grey. Orchitis (right) appeared on the 17th day, scrotal edema (right) on the 26th day, followed by massive nodular orchitis. No metastatic lesions were observed.

Rabbit No. 20.—Flemish cross. Orchitis (right) appeared on the 19th day, scrotal edema (right) on the 29th day, metastatic orchitis (left) on the 35th day, and a scrotal chancre (right) on the 66th day. A skin lesion appeared on the right foot on the 49th day. Scrotal chancre appeared on the left side on the 84th day and a skin lesion appeared on the left foot at the same time.

Rabbit No. 21.—Belgian cross. Orchitis (right) appeared on the 10th day, scrotal edema (right) on the 17th day, and metastatic orchitis (left) on the 38th day. Skin lesions appeared on the left foot on the 49th and 60th days. Scrotal chancre (right) developed on the 66th day.

Rabbit No. 22.—Black and Flemish cross. Orchitis (right) appeared on the 10th day, scrotal edema (right) on the 21st day, and metastatic orchitis (left) on the 38th day. Skin lesions appeared on the right foot on the 56th day, and on the left foot on the 64th day; periosteal lesion of fifth left metatarsal on the 60th day. Phlyctenular lesions appeared on the sclerocorneal margin of the left eye at the same time.

Rabbit No. 23.—Greyish brown. Orchitis (right) appeared on the 10th day, scrotal edema (right) on the 27th day, and metastatic orchitis (left) on the 46th day. No other generalized lesions occurred.

Rabbit No. 24.—Brown. Orchitis (right) appeared on the 17th day, scrotal edema on the 19th day, and metastatic orchitis (left) on the 54th day. Skin lesions appeared on the left foot on the 49th and 53rd days, on the right foot on the 55th day, and at the base of the tail on the 60th day. Fusiform swelling of the shaft of the fifth right metatarsal appeared on the 45th day.

Recapitulation.—Of the seven adult males which were inoculated intratesticularly, six, or 85.7 per cent, showed metastatic orchitis within a period of 41.5 days, and five, or 71.4 per cent, had metastatic lesions of other structures (skin and bone) within an average time interval of 54.7 days.

Group E.

This group comprised five adult males which were inoculated intratesticularly with 0.1 cc. of testicular emulsion containing treponemata. Only one testicle was inoculated. On the 11th day following inoculation, when lesions could be detected by clinical examination in all of the animals, the entire right testicle was removed under ether anesthesia and the wound closed with sutures. The individual protocols follow.

Rabbit No. 25.—Albino. Orchitis (right) appeared on the 10th day. Castration (right) on the 11th day. Recurrent lesion of the stump on the 38th day, later becoming a chancre; metastatic epididymitis (left) on the 53rd day. Skin lesions appeared on the right foot on the 84th day and on the left foot on the 90th day.

Rabbit No. 26.—Brown and Maltese cross. Orchitis (right) appeared on the 10th day. Castration (right) on the 11th day. Recurrent lesion of stump on the 38th day, later developing into a chancre. Metastatic orchitis (left) on the 40th day. No other generalized lesions were noted.

Rabbit No. 27.—Orchitis (right) appeared on the 10th day. Castration (right) on the 11th day. Some induration of the stump appeared but no marked lesion developed. Metastatic orchitis (left) appeared on the 50th day, lesion of nasal bones on the 70th day, and a skin lesion of the right foot on the 85th day.

Rabbit No. 28.—New Zealand red. Orchitis (right) appeared on the 10th day. Castration (right) on the 11th day. No metastatic lesions of any sort were observed, nor were there any recurrences at the operative site.

Rabbit No. 29.—Brown. Orchitis (right) appeared on the 10th day. Castration (right) on the 11th day. Recurrence at the operative site on the 29th day. Metastatic orchitis (left) on the 42nd day. Periosteal bone lesions appeared on the fifth right metatarsal on the 52nd day, on the left ulna on the 53rd day, and on the nasal bones on the 63rd day.

Recapitulation.—Of the five adult males which were inoculated intratesticularly and the primary focus removed later by castration, four, or 80 per cent, showed a metastatic orchitis within a period of 46.7 days, and in three, or 60 per cent, metastatic skin or bone lesions made their appearance within 90 days. In this group the metastatic lesions were slower in making their appearance than in the control group (Group D), in which there was no attempt made to interfere with the course of the primary focus. The ultimate incidence of generalized lesions was approximately the same in the two groups. In three of the five castrated animals, pronounced recurrent lesions developed in the operative wound. In a fourth there was a similar although less marked recurrence.

Group F.

This group comprised six adult males. All of the animals in the group were inoculated by implantation, within the right scrotal sac, of bits of syphilitic testicular material containing treponemata. The individual protocols follow.

Rabbit No. 30.—Grey. Scrotal chancre appeared on the 10th day, orchitis (right) was detected on the 31st day. Skin lesions appeared on the left foot on the 49th day, and on the right foot on the 60th day. Periosteal lesions of both radii were observed on the 52nd day, and of the right ulna on the 66th day. The chancre began to heal spontaneously before the 90th day.

Rabbit No. 31.—Albino. Scrotal chancre developed by the 10th day, a skin lesion appeared on the left foot on the 70th day. The chancre healed spontaneously before the 90th day.

Rabbit No. 32.—Albino. Scrotal chancre developed on the 10th day. Metastatic orchitis (left) appeared on the 52nd day, and on the 60th day a subcutaneous nodule of uncertain nature appeared. The chancre, after attaining an enormous size, began to heal before the 90th day.

Rabbit No. 33.—Brown. Scrotal chancre developed by the 10th day; skin lesions appeared on both feet. The chancre healed spontaneously before the 85th day.

Rabbit No. 34.—Brown. Two scrotal chancres developed by the 17th day, one at the site of implant, the other at the scrotal incision. Metastatic epididymitis (left) was noted on the 64th day. No other metastatic lesions were observed, while both chancres healed spontaneously by the 66th day.

Rabbit No. 35.—Belgian cross. Two scrotal chancres developed by the 10th day, one at the site of the implant, the other at the scrotal incision. They coalesced, forming a single chancre by the 20th day. Metastatic orchitis (left) appeared on the 46th day, skin lesions on the left foot on the 49th day, and on the right foot on the 60th day. A lesion of the nasal bones was observed on the 60th day. The chancre, after regressing appreciably, relapsed and was increasing in size at the end of the experiment.

Recapitulation.—Of the six adult males which received implantations of syphilitic testicular material in the scrotum, all developed typical chancres which healed spontaneously. In three, or 50 per cent, metastatic orchitis or epididymitis made its appearance within an average time interval of 54.9 days; and five, or 83 per cent, showed metastatic lesions of other structures in an average of 51.6 days.

GENERAL SUMMARY.

For convenience the results are summarized in tabular form. Table I shows a comparison of the primary reaction in the groups of female and male rabbits inoculated intradermally. In Table II the incidence of metastatic lesions is shown in the various groups of animals. In Table III are shown the character and date of appearance of the generalized lesions (exclusive of orchitis) in the various groups of animals.

The results presented in Tables I, II, and III, together with a study of the individual protocols, permit the following general statements to be made relative to the effect of the factors studied upon the course of experimental syphilis in the rabbit.

Sex.—In the group of females inoculated intradermally the lesions at the site of inoculation were in general less marked than in the group of males similarly inoculated. Moreover, they attained their maximum size earlier and began to recede earlier than was the case in the males. In none of the females was there clinical evidence of the production of generalized lesions, while in the corresponding group of males metastatic lesions of skin or bones detectible by clinical examination occurred in one instance, or 14.3 per cent. In both groups there was wide variation in the character of the initial reaction in individual rabbits and in the time required for it to reach its maximum size.

TABLE I.

Comparison of Primary Reactions in Male and Female Rabbits Inoculated Intradermally with Treponema pallidum.

Group.	Rabbit No.	Character of primary lesion.	Incubation period.	Maximum diameter of primary lesion.	Time of appearance of maximum size of primary lesion.
			days	*mm.*	*days*
A.	1	None.			
5 females.	2	Indurated papule.	11	4	15
	3	Transient indurated area.	10	1	10
	4	Chancre.	19	10	52
	5	Indurated papule.	10	8	21
Average......		.. . .	10	4	19.6
B.	6	Transient indurated area.	31	1	31
7 males.	7	Three indurated papules.	31	5	?*
	8	Chancre.	10	8	74
	9	"	6	15	74
	10	"	10	18	?*
	11	"	10	10	61
	12	Indurated papule.	10	5	19
Average........			15.4	9	

* Lesion still increasing in size at the end of the experiment.

Age.—In the five young males inoculated intratesticularly the disease was not greatly different from that observed in the older males similarly inoculated. The initial reactions were slightly slower in making their appearance, but the magnitude of the reaction, as judged by enlargement of the testicles, was somewhat greater. The percentage incidence of metastatic orchitis was almost the same in the two groups. The younger animals showed a slightly greater incidence of generalized lesions involving structures other than the testicles, although the difference was insignificant, but the lesions were smaller, fewer in number, and confined entirely to the skin, and there was no instance of severe generalized lesions. Moreover, the occurrence of metastatic lesions (skin) in the younger animals was definitely delayed.

Method of Injection.—In the animals inoculated by the intratesticular route the development of the primary reaction and the incidence of generalized lesions involving skin and bone were much greater than in the animals inoculated intradermally. Thus, of the latter group only one, or 14.3 per cent, showed a secondary metastatic lesion (skin), while in the group inoculated by the testicular route the incidence of metastatic skin and bone lesions was as high as 71.4 per cent. The difference in the character of the disease exhibited by these two groups of animals inoculated in a different manner is quite striking. Intratesticular inoculation produced a much more violent local reaction and a much greater incidence of generalized lesions than did intradermal inoculation.

TABLE II.

Incidence of Metastatic Syphilitic Lesions in Various Groups of Rabbits.

Group and method of inoculation.	No. inoculated.	Metastatic orchitis.			Generalized lesions (bone, skin, eye).		
		No.	Per cent.	Average time of appearance.	No.	Per cent	Average time of appearance.
Intradermal inoculation.							
				days			*days*
A. Adult females.	5				0	0	
B. Adult males.	7	3	42.8	87	1	14.3	84
Intratesticular inoculation.							
C. Young males.	5	4	80	43.7	4	80	64
D. Adult males, not castrated.	7	6	85.7	41.5	5	71.4	54.7
E. " " castrated.	5	4	80	46.7	3	60	69
Scrotal implantation.							
F. Adult males.	6	3	50	54.9	3	50	51.6

Effect of Castration.—In the group of animals with unilateral orchitis in which the infection was allowed to run its course without any attempt at suppression by removal of the initial focus, metastatic lesions appeared more promptly and in slightly greater incidence than

TABLE III.

Character and Date of Appearance of Generalized Syphilitic Lesions (Exclusive of Orchitis) in Various Groups of Rabbits.

Group.	Rabbit No.	Generalized syphilis (exclusive of orchitis and lymphadenitis).	Lesions in order of their appearance.	Recurrence.
Intradermal inoculation.				
			days	
A.	1	—		
5 females.	2	—		
	3	—		
	4	—		
	5	—		
B.	6	—		
7 males.	7	—		
	8	—		
	9	—		
	10	+++	S(89), PB(89), PB(89)	
	11	—		
	12	—		
Unilateral testicular inoculation.				
C.	13	+	S(66)	
5 young males.	14	++	S(62, 73)	
	15	+	S(60)	
	16	—		
	17	+	S(70)	
D.	18	+	S(62)	
7 males.	19	—		
	20	++	S(49, 84)	
	21	++	S(49, 60)	
	22	++++	S(56), PB(60), E(60), S(64)	
	23	—		
	24	+++++	S(49), PB(52), S(53, 55, 60)	

E indicates eyes; S, skin; PB, periosteum and bone.

TABLE III—*Concluded.*

Group.	Rabbit No.	Generalized syphilis (exclusive of orchitis and lymphadenitis).	Lesions in order of their appearance.	Recurrence.
Unilateral testicular inoculation, castration.				
E. 5 males.	25	++	S(84, 90)	+
	26	—		+
	27	++	PB(70), S(85)	±
	28	—		—
	29	+++	PB(52, 53, 63)	+
Scrotal implantation.				
F. 6 males.	30	++++	S(46), PB(52), S(60), PB(66)	
	31	+	S(70)	
	32	±	S(60)?	
	33	+	S(60)	
	34	—		
	35	+++	S(49), PB(60), S(60)	

in the group of animals in which the initial local reaction was suppressed by removal of the focus itself through castration. The percentage differences are not very great, but they are constantly in favor of the first group, as is the time interval of appearance. This experiment is not in strict accord with similar experiments observed by Brown and Pearce (3) in the past, and while the reasons for this difference are not clear the fact may be noted that the incidence of generalized lesions in uncastrated animals is somewhat higher than that reported by these authors, also that there was a high percentage of recurrence of primary lesions.

DISCUSSION.

The results are sufficiently clear-cut to permit of a generalization on several points. The difference in the character of the initial local response and in the subsequent production of generalized lesions, exhibited by the methods of intradermal and intratesticular inoculation, is striking, and can scarcely be explained upon the basis of biological variation in groups of animals. One is forced to conclude either that the superficial layers of the skin of the rabbit (the scrotum excluded) constitute a relatively unfavorable site for the active proliferation of the treponemata when small amounts are introduced, or else that the defensive mechanism at this spot is brought to such a level as almost completely to suppress the initial reaction, without, however, preventing general invasion of the body with lymph gland involvement. By contrast the testicle is a much more favorable site for the setting up of a clinically recognizable lesion. Whether this is due to the presence in the testicle of an abundance of substances favoring growth of the treponemata, or to the absence of conditions that favor the operation of factors of resistance it is impossible to say. Perhaps both influences may be at work side by side. The successful production of primary reactions in the scrotum by implantation of bits of tissue containing treponemata does not in our opinion alter the generalization that the primary reactions obtained by testicular inoculation are greater than those obtained by intradermal inoculation. In the case of the scrotal implantations the inoculum was of necessity much larger than that employed for intratesticular and intradermal inoculation.

The difference in the behavior of the females and the males toward intradermal inoculation is of interest. In the former the local reaction was much less prominent than in the males, attained its maximum size more rapidly, and cleared up more promptly. None of the five females developed generalized lesions of other structures, whereas in two of the seven males an orchitis developed, and in a third there was evidence of generalization of the virus in the shape of skin and bone lesions. The behavior of these five females was different from that of eight normal females formerly reported in a paper (4) dealing with the reaction of pregnant females to inoculation with *Treponema pallidum*. In that paper it was stated that non-pregnant female rabbits reacted to intradermal inoculations of the vulva with the prompt development of characteristic indurated lesions at the site of inoculation. Pregnant animals, however, when inoculated under similar conditions were found to react either not at all or with much less marked clinical phenomena. In the five non-pregnant animals reported upon in this paper the reaction to intradermal inoculation approached more closely that exhibited by the pregnant animals in the foregoing paper. This discrepancy in results is probably explainable on the basis of a difference in virulence or a difference in the severity of the prevailing disease since a difference of the same character was observed in the behavior of the male animals of the two series.

Whatever its explanation, it serves to show that the behavior of any one group of rabbits toward infection with *Treponema pallidum* is by no means indicative of what may be expected subsequently in another group of animals even if infected with the same strain. In our opinion this point has not received sufficient attention, particularly from some of those workers in experimental syphilis who have been attempting to classify strains on the basis of the primary reactions exhibited by the rabbit host. Many of the variations in primary reactions that have been proposed as characteristics for differentiating one strain from another were, to judge from their description, no more marked than those exhibited by the group of female rabbits reported upon in this paper.

It is of interest, also, that in all of the five females that were inoculated intradermally the organisms were able to invade the body and localize in distant lymph nodes in spite of the relatively slight local lesion at the portal of entry or the total absence of the same. This observation parallels similar observations by Neisser in monkeys and is in agreement with previous work (5) in which it was demonstrated that *Treponema pallidum* might make its way through the genital mucosa of rabbits and invade the body, without calling forth any reaction at the site of inoculation.

With respect to the factor of age, the results were not sufficiently clear-cut to warrant any broad generalization. In general, the younger animals reacted with a more violent initial lesion, and were apparently able to keep the subsequent manifestations under control for a slightly longer period of time, as well as to prevent the occurrence of any severe generalized lesions. There was a rather striking uniformity in the manner in which the individual animals in this group reacted to the infection at the site of inoculation.

The effect of removal of the initial focus on the subsequent production of generalized lesions, which in former experiments by Brown and Pearce (3) resulted in a greater incidence of the latter, was not observed in the experiments herein reported. In those animals in which the primary reaction was removed by castration the incidence of generalized lesions was no greater and the time of occurrence was distinctly postponed. The former experiments seemed to point definitely to an influence on the course of the infection exerted by the initial lesion. Procedures calculated to suppress the initial lesion (removal, insufficient treatment with arsenicals) resulted in a greater incidence, within certain time limits, of generalized lesions in the animals in which the interference with the initial lesion was instituted than in the controls. Upon this basis an hypothesis spoken of as a "law of inverse ratio" was formulated. The experiments herein described would seem to indicate that under conditions as yet unknown there may be exceptions to this principle.

CONCLUSIONS.

1. Rabbits inoculated intradermally with active syphilitic virus are within specified time limits less prone to develop generalized syphilis recognizable by clinical methods of examination than those inoculated intratesticularly.
2. The primary reaction to intradermal inoculation of syphilitic virus was distinctly less marked in females than in males.
3. Young rabbits react to an intratesticular inoculation of the syphilitic virus with a slightly more marked initial lesion than do older animals similarly inoculated, and tend to postpone slightly the appearance of subsequent generalized lesions, which are less severe in character and extent.
4. Suppression of the initial reaction by excision does not always lead to an increased incidence or early appearance of subsequent generalized lesions within an observation period of 90 days.
5. In rabbits inoculated intradermally in which no reaction or only a slight reaction developed at the site of inoculation, general invasion of the body by the treponemata may take place, as evidenced by positive lymph node transfer, although no generalized lesions may be observable.

The experiments reported in this paper were carried out at the suggestion and under the supervision of Dr. Wade H. Brown. Grateful acknowledgement is hereby made to Dr. Brown and to Dr. Louise Pearce for their interest and assistance in prosecuting the work.

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DIFFERENTIATION OF OXYHEMOGLOBINS BY MEANS OF MUTUAL SOLUBILITY TESTS.

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In recent papers[1] the authors were able to show by serological tests that hemoglobin exhibits species specificity to a high degree. It seemed of advantage, however, to demonstrate these differences, undoubtedly chemical in character, by some independent method. The solubility of an individual protein itself might be taken as a criterion of chemical entity, as has been suggested recently by Cohn.[2] As, however, this method requires the attainment of absolute purity, aside from the possibility that several substances might have the same solubility within the limits of error, another method was sought.

Of the possibilities which suggested themselves, a promising one seemed the use of the well known fact that the solubilities of substances which do not react with each other are additive. As a consequence it would be expected that if two samples of oxyhemoglobin were different each would dissolve in a saturated solution of the other as in pure water itself. On the other hand, if two preparations were identical, a saturated solution of one would obviously be saturated to both.

As the method used in the present work was a comparative one, extreme accuracy was not aimed at. Hence the solubility determinations were carried out simply at room temperature. 1 cc. samples of the solutions were used, and the content in hemoglobin was determined by drying to constant weight *in vacuo* at 40–50°C.

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[1] Landsteiner, K., *Verslag Kon. Akad. van Wetensch. te Amsterdam*, 1921, xxix, 1029. Heidelberger, M., and Landsteiner, K., *J. Exp. Med.*, 1923, xxxviii, 561.

[2] Cohn, E. J., *J. Gen. Physiol.*, 1921–22, iv, 697.

The preparations of oxyhemoglobin were made according to the method recently published by one of us.[3] In each case freedom from salts was controlled by conductivity measurements, and in several instances electrometric pH determinations were run on the solutions in order to make certain that the observed solubility effects were not due to differences in the pH of the solutions.

As an example of the manipulation used the following experiment (No. 2) is described.

Recrystallized horse and dog oxyhemoglobin were used. The conductivities of the aqueous solutions were respectively 4.3×10^{-6} and 2.7×10^{-5}. Each oxyhemoglobin in the form of a moist crystalline paste was mixed with water in a tube and shaken mechanically for ¾ of an hour. That equilibrium was approximately attained within this period was shown by a determination of the hemoglobin in solution, and a comparison of the amount found with the hemoglobin content after the next shaking, the latter value being the one given at the head of each column in the tables. Usually a slight increase was noted. At the end of the period of shaking the tubes were centrifuged and the supernatant liquid from each was poured in equal parts into two tubes. To one of these was added more of the homologous protein, while to the other the oxyhemoglobin of the other species was added. The four tubes were now shaken again for ½ hour and centrifuged. In every case before taking the analytical samples the solutions were filtered through small analytical filters in order to hold back any crystal fragments. 1 cc. portions of each were now dried and the residue was weighed.

The results are given in the following tabulations, in which the figures represent the weight of the residue from 1 cc. in gm. In each case the value after "difference" should approximate that at the head of the opposite column.

Experiment 1.

Dog + dog	0.0275	Horse + horse	0.0080
Dog + horse	0.0384	Horse + dog	0.0302
Difference	0.0109	Difference	0.0222

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[3] Heidelberger, M., *J. Biol. Chem.*, 1922, liii, 31.

Experiment 2.

Conductivities: horse HbO_2 4.3×10^{-5}; dog HbO_2 2.7×10^{-5}.

Dog + dog	0.0238	Horse + horse	0.0158
Dog + horse	0.0372	Horse + dog	0.0417
Difference	0.0134	Difference	0.0259

Experiment 3.

Dog + dog	0.0260	Horse + horse	0.0151
Dog + horse	0.0395	Horse + dog	0.0400
Difference	0.0135	Difference	0.0249

Experiment 4.

Conductivities: horse HbO_2 3.2×10^{-5}; dog HbO_2 3.5×10^{-5}; guinea pig HbO_2 2.9×10^{-5}.

pH electrometric,[*] respectively: 6.69, 6.82, 6.76.

Dog + dog	0.0256	Horse + horse	0.0107
Dog + horse	0.0356	Horse + dog	0.0293
Difference	0.0100	Difference	0.0186
Dog + dog	0.0256	Guinea pig + guinea pig	0.0046
Dog + guinea pig	0.0274	Guinea pig + dog	0.0318
Difference	0.0018[†]	Difference	0.0272
Horse + horse	0.0107	Guinea pig + guinea pig	0.0046
Horse + guinea pig	0.0140	Guinea pig + horse	0.0159
Difference	0.0033	Difference	0.0113

Experiment 5.

Conductivities: guinea pig 2.8×10^{-5}; rat 3.8×10^{-5}.

pH electrometric, respectively: 6.69, 6.95.

Guinea pig + guinea pig	0.0040	Rat + rat	0.0039
Guinea pig + rat	0.0071	Rat + guinea pig	0.0086
Difference	0.0031	Difference	0.0047

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[*] The writers are indebted to Dr. A. B. Hastings for these determinations.

[†] That this value is too low is probably due to the fact that, as mentioned above, saturation was not always complete at the end of the first shaking. This would have little influence except in a case such as this, in which the solubility of the second type of hemoglobin is much less than that of the first.

It will be seen from the above experiments that in four of the species tested, differences between the oxyhemoglobins were indicated by the increase in the dissolved hemoglobin. This increase was in most cases not far from the solubility of the added type of oxyhemoglobin in water alone. It thus appears that the method is capable of bringing to light chemical differences between the oxyhemoglobins of not too closely related species. The trial of this procedure in the study of other proteins would therefore seem desirable.

As a control on the method Experiment 6 was performed. In this experiment two different lots of horse oxyhemoglobin were compared as before. As was to be expected the result was negative.

Experiment 6.

Comparison of two different preparations of horse oxyhemoglobin (I and II). Conductivities: I 2.2×10^{-5}; II 3.3×10^{-5}.

I + I	0.0120	II + II	0.0107
I + II	0.0118	II + I	0.0107

Experiment 7.

Conductivities: horse HbO_2 3.3×10^{-5}; donkey 1.5×10^{-5}.

Horse + horse	0.0166	Donkey + donkey	0.0086
Horse + donkey	0.0139	Donkey + horse	0.0128
Difference	−0.0027	Difference	0.0042

In Experiment 7 oxyhemoglobins of two closely related species, namely the horse and the donkey, were investigated. This case was of special interest in that our serological tests indicated so slight a dissimilarity as to render difficult a serological differentiation. In the present series of experiments, also, this case differed from the others, as no addition of solubilities took place. While it would seem that the final values were between the solubilities of the two preparations, too much stress cannot be laid upon this point, as the absolute value of the solubility of horse oxyhemoglobin varied in the different experiments, and the cause of this variation has still to be determined.

Since, from general considerations, identity of the two proteins is improbable, it would appear that the two oxyhemoglobins behave as isomorphous compounds. It is interesting that such a relationship should be found in a case in which the proteins appear compatible *in vivo,* as is shown by the ability to cross these animals. In this connection we must mention the comprehensive work of Reichert and Brown[4] on the crystallography of hemoglobin with respect to species differences. Its possible bearing upon the point just mentioned has been stated by Loeb.[5]

CONCLUSIONS.

1. The rule of addition of solubilities is applicable to the differentiation of the oxyhemoglobins of not too closely related species.

2. The oxyhemoglobins of the horse, dog, rat, and guinea pig show differences when tested by this method. The oxyhemoglobins of the donkey and horse show a similarity which is best explained by the assumption of isomorphism.

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[4] Reichert, E. T., and Brown, A P., *Carnegie Inst. Washington, Pub. 116,* 1909.

[5] Loeb, J., *Science,* 1917, xlv, 191.

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CANCER ET INFECTION RENALE A COCCIDIES CHEZ LA SOURIS.

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Au cours de recherches que j'effectuais à Copenhague, dans le laboratoire du P^{r} Fibiger, j'eus l'occasion d'observer l'été dernier, dans les reins de Souris splénectomisées et badigeonnées au goudron de houille depuis 4 mois et plus, des formes parasitaires que j'eus de la peine à identifier. Pour diverses raisons, je dus alors abandonner ces recherches. J'ai pu les reprendre ici au Rockefeller Institute, à New York, dans les laboratoires du Dr. Murphy. La note présente résume les premiers resultats.

Ayant eu l'heureuse fortune de découvrir des reins frais, abondamment parasités, je pus assez aisément déterminer que j'avais affaire à un parasite de la meme espèce, sinon le même que celui décrit par Th. Smith en 1889 et 1904 (1). Cet auteur a bien déterminé une partie du cycle de vie de cette coccidie nouvelle, parasitant les cellules des tubes rénaux, il l'a appelée du nom de *Klossiella muris*. La description qui suit est celle du parasite observé par moi; elle diffère légèrement de celle de Smith, surtout quant au nombre des sporoblastes-filles et à celui des sporozoïtes. Le cycle ordinaire que l'on constate dans le rein est le cycle sporulé. Les sporozoïtes (sporontes), provenant probablement du sang, passent à travers les glomérules, tombent dans la lumière des tubes rénaux, pénètrent dans le protoplasme des cellules rénales, et s'y développent. Ces sporozoïtes libres ont une forme arrondie et atteignent à peine, en plein développement, le volume d'un globule rouge. Une fois entrés dans la cellule, leur protoplasme s'agrandit, leur noyau qui, primitivement, était représenté

(1) T. Smith. *Journ. Comp. Med. and Surg.*, 1889, t. x, p. 211.—T. Smith et H.-P. Johnson. *Journ. of Exper. Med.*, 1904, t. vi, p. 303.

par une minuscule tache de chromatine, se divise en un certain nombre (6–10) de masses semblables. Le protoplasme se fragmente à son tour et amène la formation d'autant de sporoblastes-filles. Le tout réuni (sporoblastes-mère) est représenté par une masse unique sphéroïdale de 30 μ, ou plus, de diamètre, toujours contenue dans la cellule rénale qui l'enveloppe d'une mince bande protoplasmique. A ce stade, cette cellule ressemble ordinairement à une massue dont la tête contenant le sporoblaste-mère ferait hernie dans la lumière du tube contourné et le manche, représenté par un mince pédicule protoplasmique, se maintient attaché a la membrane basale du tube rénal. Le noyau de cette cellule, fortement aplati, est refoulé en un point de la périphérie. Bientôt, le noyau de chacun de ces sporoblastes-filles se divise à son tour en 10 à 20 petites masses chromatiques, la division protoplasmique suit de près et amène la formation d'autant de sporozoïtes. Chaque sporoblaste-fille s'entoure alors d'une membrane hyaline: dès lors une spore de 15 μ de diamètre environ est formée. Comme chaque sporoblaste-fille donne naissance à une spore, chacun des parasites initiaux engendre environ 200 sporozoïtes nouveaux. Les spores passent dans l'urine et vont probablement, par là, infecter d'autres animaux d'une façon totalement inconnue. Après Smith, nous avons, de plus, observé dans les glomérules, une forme parasitaire dont le rôle est difficile à déterminer, mais qui représente probablement le cycle schizogonique. On ne la rencontre que chez les animaux très fortement infectés. Cette description trop courte est forcément incomplète et obscure. Nous la publierons ailleurs avec tous les détails voulus.

Quoi qu'il en soit, Th. Smith dit n'avoir rencontré ce parasite que par hasard, toujours chez des animaux adultes, paraissant normaux et ayant cohabité en grand nombre pendant longtemps; il semble néanmoins avoir très mal réussi à transmettre l'infection par voie digestive.

Nous l'avons dit, nos propres recherches ne sont pas terminées, mais nous pouvons signaler dès à présent que l'on rencontre ces parasites avec une fréquence remarquable chez les animaux porteurs d'un cancer avancé et, exceptionnellement, chez des animaux supposés normaux, du moins chez ceux que nous avons examinés.

Voici quelles sont les proportions exactes d'animaux parasités que nous avons observés dans les diverses catégories étudiées jusqu'ici:

	Nombre d'animaux examinés	Nombre d'animaux parasités	Pourcentage.
Animaux normaux d'âges divers	90	2	2.2
Animaux porteurs d'un cancer spontané	21	17	79 9
Animaux porteurs d'un cancer du goudron ayant donné des métastases.......	2	2	Cas trop peu nombreux.
Animaux porteurs d'un cancer du goudron au début.	4	0	Cas trop peu nombreux.

Toutes ces recherches ont été faites sur des coupes microscopiques de reins inclus à la paraffine et débités en coupes de 5–10 μ d'épaisseur. Nous avons employé des colorations diverses, la meilleure est l'hématoxyline-érythrosine-safran de Masson.

Nos investigations continuent dans de nombreuses directions, de façon à essayer de déterminer les conditions favorisant l'infection des animaux par ce parasite. Nous communiquerons plus tard nos résultats définitifs. Dès à présent, sans vouloir aucunement établir de relations de causalité entre la présence de ce parasite dans le rein et celle d'un cancer en une partie quelconque du corps, il nous semble intéressant de signaler ce fait curieux que l'état cancéreux d'un individu serait un état spécialement favorable au développement de cette coccidie.

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A MICRO METHOD FOR THE DETERMINATION OF THE HYDROGEN ION CONCENTRATION OF WHOLE BLOOD.

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The electrometric and colorimetric methods for determining the blood reaction in use at present require such quantities of blood that they are not applicable for use with small laboratory animals.

The method described below, requiring only small quantities of blood, is a slight modification of the method recently described by Cullen.[1]

Experiment.

30 drops of a 0.03 per cent solution of phenol red are added to 50 cc. of a 0.9 per cent solution of sodium chloride and adjusted to pH 7.3. 5 cc. portions of this solution are placed in tubes with a diameter of 16 mm. and covered with paraffin oil.

The blood is drawn from the heart or a vein directly into a 1 cc. pipette graduated to hundredths, by attaching a needle with a short rubber tube to the pipette.

The needle and rubber are then detached and 0.25 cc. of the blood is run under the oil into one of the tubes containing the saline indicator solution. The blood and solution are thoroughly mixed by stirring carefully with a clean glass rod.

The tube is centrifuged for 10 minutes, completely throwing down the red corpuscles, and is then placed in a comparator block and the pH determined by matching to the nearest color standard and applying corrections as described by Cullen. The standard color tubes are 16 mm. in diameter and contain 5 cc. of Sörenson's standard phosphate solutions ranging in steps of 0.05 pH from pH 7.00 to 7.80.

For the comparison pH determinations were made simultaneously on the same sample of blood, using both the above modification and the original method as described by Cullen.[1]

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[1] Cullen, G. E., *J. Biol. Chem.*, 1922, lii, 501.

Comparison of pH Values as Determined by the Two Methods.

No.	Whole blood.	Plasma.	Deviation.
Rabbits (arterial blood).			
1	7.29	7.29	0.00
2	7.29	7.30	−0.01
3	7.32	7.34	−0.02
4	7.42	7.42	0.00
5	7.29	7.31	−0.02
6	7.49	7.48	+0.01
7	7.32	7.35	−0.03
8	7.53	7.53	0.00
9	7.48	7.50	−0.02
10	7.48	7.46	+0.02
11	7.41	7.41	0.00
12	7.46	7.47	−0.01
13	7.42	7.42	0.00
14	7.43	7.41	+0.02
15	7.46	7.43	+0.03
16	7.45	7.43	+0.02
17	7.43	7.41	+0.02
18	7.26	7.25	+0.01
19	7.33	7.32	+0.01
20	7.30	7.29	+0.01
21	7.32	7.33	−0.01
22	7.33	7.33	0.00
23	7.31	7.32	−0.01
24	7.32	7.33	−0.01
Rabbits (venous blood).			
1	7.29	7.30	−0.01
2	7.33	7.33	0.00
3	7.34	7.37	−0.03
4	7.35	7.37	−0.02
5	7.32	7.33	−0.01
6	7.29	7.30	−0.01
Guinea pigs (arterial blood).			
1	7.42	7.44	−0.02
2	7.46	7.46	0.00
3	7.44	7.44	0.00
4	7.45	7.48	−0.03
5	7.40	7.40	0.00
6	7.36	7.35	+0.01

Comparison of pH Values as Determined by the Two Methods—Concluded.

No.	Whole blood.	Plasma.	Deviation.
Guinea pigs (arterial blood)—Concluded.			
7	7.31	7.31	0.00
8	7.37	7.35	+0.02
9	7.36	7.36	0.00
10	7.33	7.34	−0.01
Guinea pigs (venous blood).			
1	7.38	7.39	−0.01
2	7.32	7.34	−0.02
3	7.46	7.44	+0.02
4	7.49	7.49	0.00
5	7.32	7.32	0.00
Human (venous blood).			
1	7.31	7.34	−0.03
2	7.29	7.30	−0.01
3	7.35	7.35	0.00

The pH as determined by the two methods agrees in most cases, the maximum deviation being 0.03 pH. The values reported above were all obtained at room temperatures between 20 and 24°C. The values of the rabbit and human bloods are corrected to electrometric pH_{38°; *i.e.*, $pH_{38^\circ} = pH_{\text{colorimetric } 20^\circ} - C$.

This method can be used in studying the changes of reaction in small laboratory animals during a course of experiments without affecting the animals by loss of blood. In addition, the total CO_2 content of the blood may also be determined, using 0.2 cc. of the excess blood in the pipette, by Van Slyke's method.[2]

SUMMARY.

A method is described for determining the reaction and the total CO_2 content of the blood using 0.6 cc. of the whole blood instead of plasma, thus rendering it possible to make repeated observations on small laboratory animals.

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[2] Van Slyke, D. D., *Proc. Nat. Acad. Sc.*, 1921, vii, 229.

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, November 1, 1923, Vol. xxxviii, No. 5, pp. 645–653.]

LOCAL RESISTANCE TO SPONTANEOUS MOUSE CANCER INDUCED BY X-RAYS.

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(Received for publication, June 27, 1923.)

Recent experimentation has tended to show that cancer is not so sensitive to radiation as earlier observers believed.[1] Although there may be some variation in the amount of x-rays required to kill various transplanted mouse[2] and rat tumors, the more careful experiments indicate that the lethal dose is rarely, if ever, within the limits of a therapeutic dose for man. On the other hand, there is no doubt that certain forms of cancer are cured by x-rays.

The recent revival of interest in x-ray therapy, due largely to the development of apparatus for generating more penetrating rays, opens up anew the question of the mode of action of this agent. If cancer is more sensitive to x-rays than normal tissue, as is generally believed to be the case, this new development is unquestionably a move in the right direction; but there is no substantial experimental basis to uphold this belief, and very good evidence to the contrary. Obviously the two facts—that cancer cells are not easily killed by x-rays, and yet that cancer may be cured by this agent—require examination if x-ray therapy is to be put on a rational basis and to be developed into a more effective form of treatment.

It has already been shown that x-rays, given over an area of skin in an erythema dose, render this area highly resistant to a subsequent inoculation with a transplantable cancer.[3] It is our opinion that this increased resistance is due to the fact that x-rays induce in this exposed area a pronounced cellular reaction of the type which under other conditions is associated with resistance to cancer. As a transplantable tumor was used for these experiments, no general deduction can be drawn as to the behavior of the spontaneous disease, and therefore it seemed advisable to determine whether the same principle was operative under the same experimental conditions when autografts of spontaneous cancer were used, thus reproducing conditions more nearly comparable to those which exist for the disease as it occurs in man.

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[1] Hill, E., Morton, J. J., and Witherbee, W. D., *J. Exp. Med.*, 1919, xxix, 89.

[2] Wood, F. C., and Prime, F., *J. Am. Med. Assn.*, 1920, lxxiv, 308.

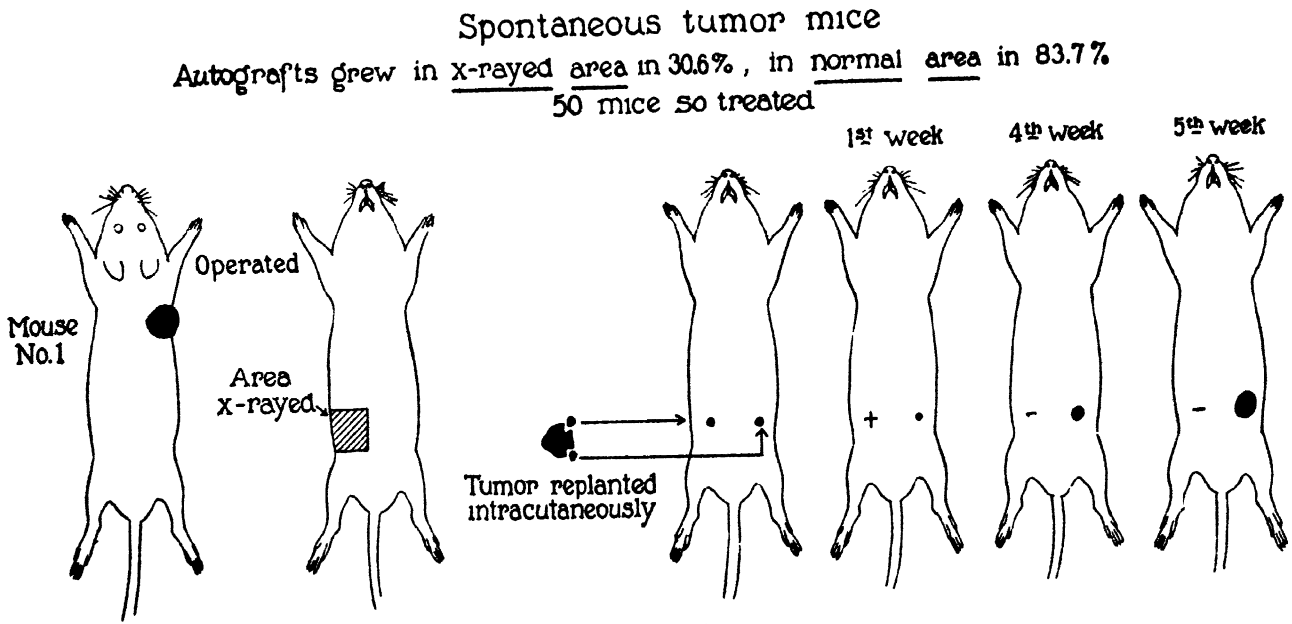
[3] Murphy, Jas. B., Hussey, R. G., Nakahara, W., and Sturm, E., *J. Exp. Med.*, 1921, xxxiii, 299

Experiment 1.—A mouse with a spontaneous mammary cancer was operated upon with removal of the tumor. With the tumor out, an area on the left flank, 12 x 15 mm. was exposed to an erythema dose of x-rays, governed by the following facts: spark-gap 3 inches; milliamperes 10; distance 6 inches; time 2½ minutes. Immediately after this treatment a small bit of the original cancer was reinoculated intracutaneously in the x-rayed area, and a like graft in the right flank, which had been protected from the x-rays (Text-fig. 1).

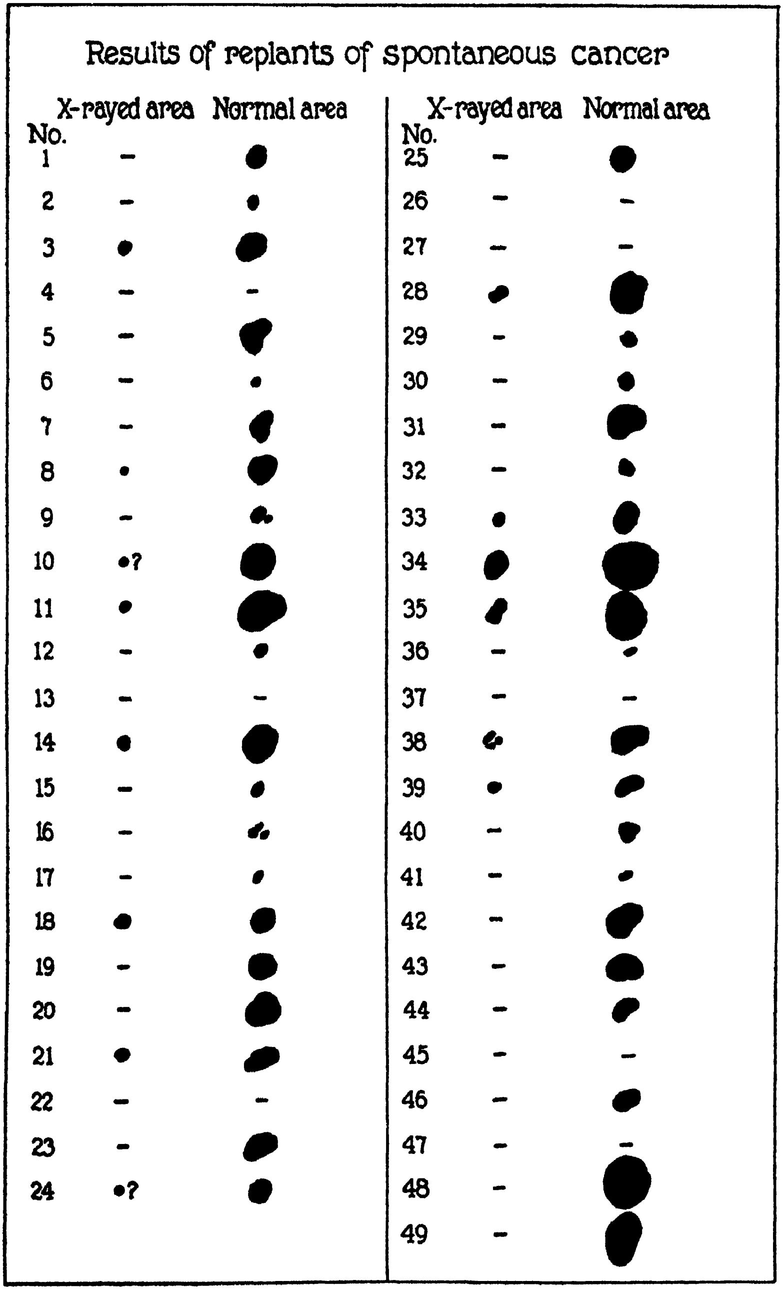
Among forty-nine mice with various types and stages of mammary tumors, subjected to this treatment, the graft inoculated in the x-rayed area failed to grow in thirty-five of the animals (71.4 per cent), while the graft in the untreated area failed in only eight (16.4 per cent) When the graft in the x-rayed area grew, it invariably progressed at a much slower rate than the corresponding graft in the normal skin, so that at the time of death of the animal it was never more than a fraction of the size of the other tumors (Text-fig. 2).

From these experiments it is evident that the local immunizing power of x-rays is just as effective against autografts of spontaneous cancers as it is against implants of a transplantable tumor. In this experiment, however, as well as in the earlier ones dealing with the transplantable tumor, the x-ray treatment at best has prevented a take or retarded the subsequent growth of the graft. It is conceivable that slightly unfavorable environmental conditions, insufficient to influence an established tumor, might be sufficient to prevent the take of a graft in which presumably the tumor cells are at a disadvantage. Will the conditions induced by x-rays be sufficiently unfavorable to influence an established tumor in the skin? The following experiment was outlined to answer this question, and also, by way of comparison, to test the direct action of x-rays on the cancer cells.

Experiment 2.—A mouse with spontaneous cancer was operated on with removal of the tumor. The tumor was then divided into two parts, and one of these subjected to an erythema dose of x-rays *in vitro.* (Spark-gap 3 inches; mili-


Spontaneous tumor mice
Autografts grew in x-rayed area in 30.6%, in normal area in 83.7%
50 mice so treated
Mouse No.1
Operated
Area x-rayed
Tumor replanted intracutaneously
1st week
4th week
5th week
+
-
-

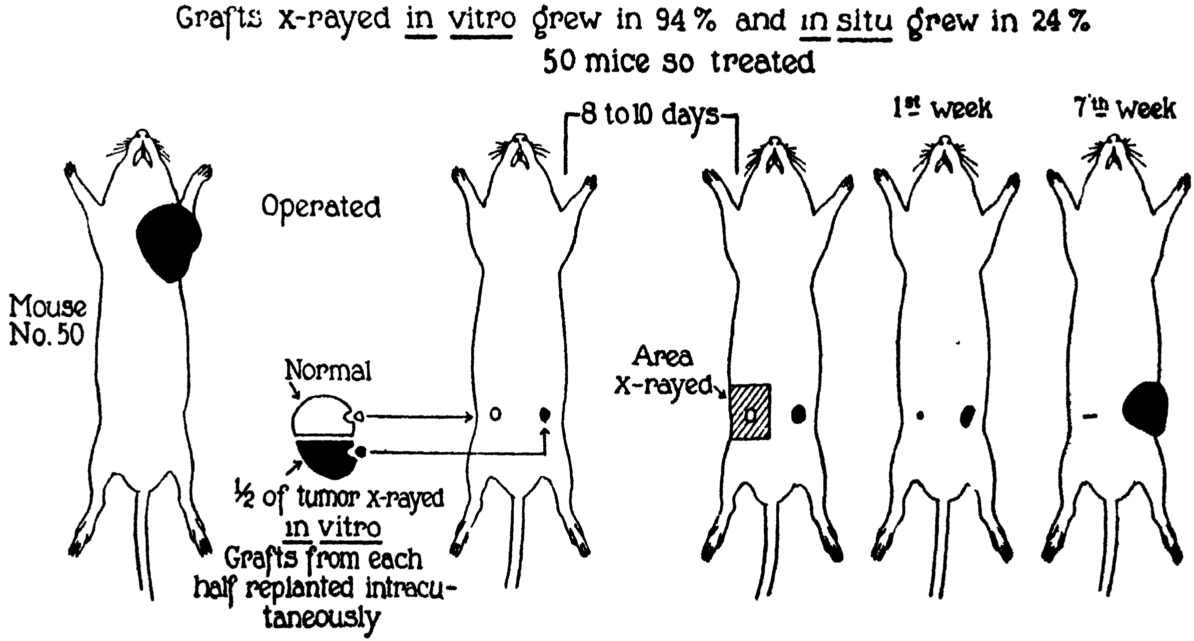
TEXT-FIG. 1.



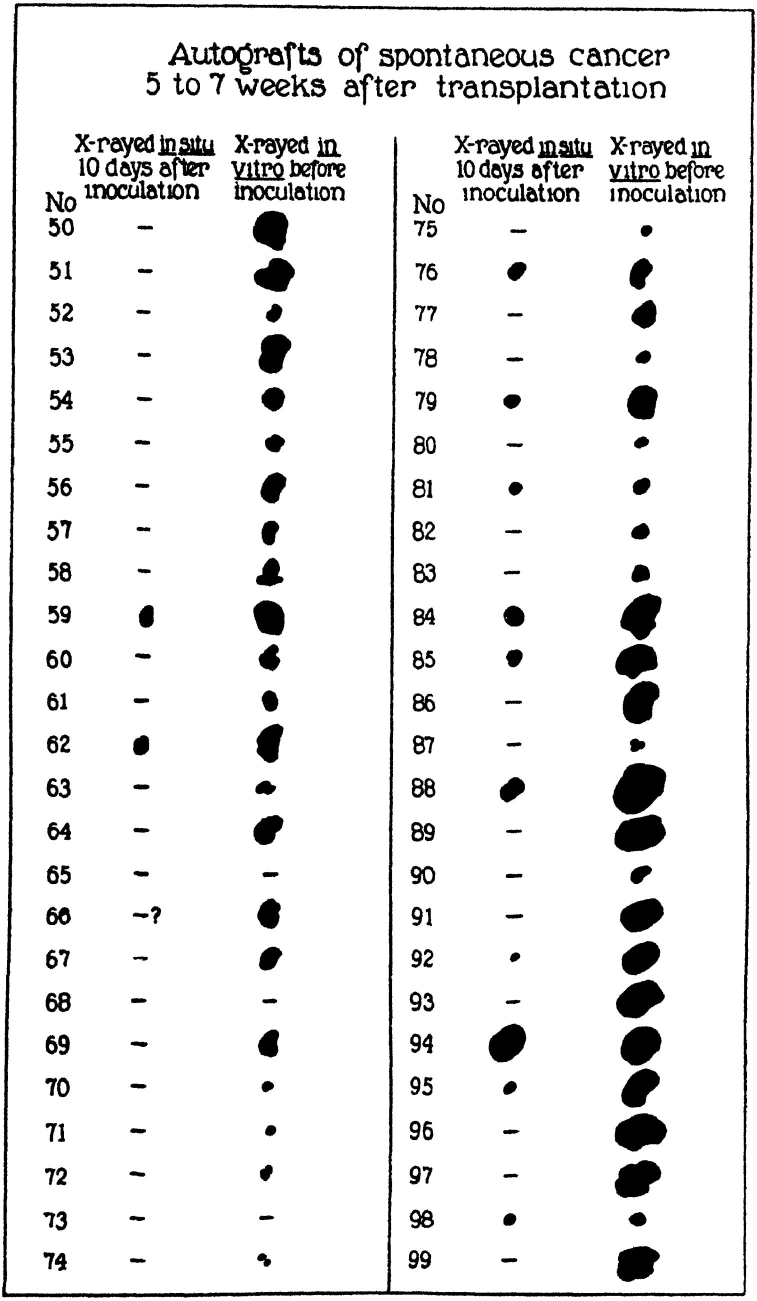
Results of replants of spontaneous cancer

No.	X-rayed area	Normal area	No.	X-rayed area	Normal area
1	–	●	25	–	●
2	–	●	26	–	–
3	●	●	27	–	–
4	–	–	28	●	●
5	–	●	29	–	●
6	–	●	30	–	●
7	–	●	31	–	●
8	●	●	32	–	●
9	–	●	33	●	●
10	●?	●	34	●	●
11	●	●	35	●	●
12	–	●	36	–	●
13	–	–	37	–	–
14	●	●	38	●	●
15	–	●	39	●	●
16	–	●	40	–	●
17	–	●	41	–	●
18	●	●	42	–	●
19	–	●	43	–	●
20	–	●	44	–	●
21	●	●	45	–	–
22	–	–	46	–	●
23	–	●	47	–	–
24	●?	●	48	–	●
			49	–	●

Text-Fig. 2.


Grafts x-rayed in vitro grew in 94 % and in situ grew in 24 %
50 mice so treated
8 to 10 days
1st week
7th week
Mouse No. 50
Operated
Normal
½ of tumor x-rayed in vitro
Grafts from each half replanted intracutaneously
Area X-rayed

TEXT-FIG. 3.


Autografts of spontaneous cancer
5 to 7 weeks after transplantation
No
X-rayed in situ 10 days after inoculation
X-rayed in vitro before inoculation
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
66
–?
67
68
69
70
71
72
73
74
No
X-rayed in situ 10 days after inoculation
X-rayed in vitro before inoculation
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99

TEXT-FIG. 4.

amperes 10; distance 6 inches; time 2½ minutes; no filter.) A small bit of this portion of the tumor, taken from the surface nearest the x-ray tube, was inoculated intracutaneously in the right flank of the original animal.[4] A graft of similar size from the untreated portion of the tumor was inoculated in the same way in the left flank (Text-fig. 3).

In the fifty mice subjected to this experiment, the grafts from the cancer x-rayed outside the body, with perhaps two exceptions, grew as rapidly as the untreated cancer, and in many cases more rapidly. After about 10 days, sometimes longer, when the new tumors had become established, the one which originated from the untreated graft was given the same dose of x-rays *in situ*, which the other tumor had received *in vitro*, the treatment including the surrounding normal skin as well as the tumor. This was followed by a prompt disappearance of the tumor in thirty-eight of the fifty animals (76 per cent) so treated, whereas the grafts from the portion of tumor x-rayed *in vitro* continued to grow in forty-seven of the fifty mice, failing in only three (6 per cent). In the twelve instances in which the tumor x-rayed *in situ* did not disappear after treatment, without exception it grew at a slower rate than the other tumor arising from the graft which had been x-rayed *in vitro* (Text-fig. 4).

There seems little doubt from the results of this experiment that a treatment dose of x-rays fails to have any appreciable direct effect on the cancer cells, yet the same dose given to a growing cancer together with the surrounding normal tissue brings about healing in a majority of cases.

There is still another point to be considered. Is it possible that tumors x-rayed *in situ* are more sensitive than those exposed *in vitro*?

Experiment 3.—As in the previous experiments, a spontaneous mouse tumor was removed at operation, and without treatment either to the tumor or the animal, small bits of the tumor were reinoculated intradermally in both flanks. After the resultant tumors were well established and growing actively, one of them was exposed *in situ* to the same dose of x-rays as that used in the preceding experiment. After the treatment this tumor was removed and again reinoculated into an unrayed area of the same animal. Forty-seven mice with spontaneous cancer received this treatment and in thirty-seven (78.8 per cent) instances the x-rayed tumor grew well in its new location.

It would seem therefore that there is no increased susceptibility of tumor cells to x-rays when treated *in situ*, and that tumor treated in such a fashion, when removed from the unfavorable environment induced by the x-rays, will grow actively when replanted in a new location on the same animal.

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[4] With the quality of x-rays used here the increased dosage due to scattering would be theoretically as great in the locality from which the graft was taken as in a tumor of the surface layers of an animal exposed to the same initial dosage.

DISCUSSION.

The fact that a large proportion of certain forms of skin cancer yield to x-ray and radium treatment is one of the chief supports for the belief that the malignant cell is more susceptible to radiation than normal tissue. An attempt has been made in the experiments reported here to analyze the mechanism by which x-rays affect the tumor lying within the skin layers. The extent to which one is justified in assuming similarities between the behavior of tissue in man and lower forms of animals is still a question, but there is little doubt that spontaneous cancer as it occurs in animals closely resembles the disease in man. It is evident from our experiments that, as far as mouse cancer is concerned, the beneficial result from x-ray therapy is due to the reaction in the normal tissues induced by the rays, not to any direct effect on the cancer cells. That this point, first brought out with a transplantable tumor, and now confirmed for the spontaneous disease, may hold true for human cancer is not improbable. Statements by Ewing,[5] based on a careful study of human material, indicate that the reaction induced in the surrounding normal tissues by x-rays or radium is of as great importance as we have shown it to be in animals. In a recent address he makes the following statements. "It is clear that the reaction of the tissues is an essential factor in the curative process. Under some circumstances, when this reaction fails, no amount of radiation succeeds in killing the tumor cells . . . . the most detailed knowledge we possess indicates clearly that the curative action is not the result of a direct effect exclusively upon the tumor cells, but involves especially a peculiar reaction of the normal or invaded tissues."

Whether the beneficial results from the use of high frequency x-rays depend on the same factors is a point as yet undetermined; but this seems not improbable, since the maximum amount of x-rays supposedly delivered to the deep tumors in such treatment is well below the

[5] Ewing, J., *Am. J. Roentgenol.*, 1922, ix, 331.

experimentally established lethal dose for cancer cells. The wave length of the rays used in deep therapy is shorter than that of those previously used, yet longer than the gamma rays of radium. Since both the relatively long x-ray waves and the short waves of the gamma ray in all probability influence cancer through the reaction induced in the normal tissue, it is not unreasonable to expect that the high frequency x-rays will eventually be found to act in the same way.

SUMMARY.

Autografts from spontaneous cancers of mice when replanted into areas previously exposed to an erythema dose of x-rays, failed to grow in the majority of instances (71.4 per cent), while similar grafts inoculated into untreated areas grew in a large proportion of the animals (83.6 per cent).

Autografts of spontaneous cancer, established and growing in the skin, disappeared in 76 per cent of animals after the tumor and surrounding tissues had been exposed to an erythema dose of x-rays, whereas other autografts of similar derivation that had been given a like dose of x-rays outside of the body and had been implanted in the same animals grew progressively in 96 per cent of instances. That this result was not due to a greater susceptibility of the cancer cells x-rayed *in situ* was shown by the fact that tumors treated *in situ* with x-rays and then replanted in an unrayed location on the same animal grew actively. Evidently the ray had done no direct damage to the cancer cells.

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ON THE PREPARATION OF DIACETONE GLUCOSE.

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(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, July 9, 1923.)

Diacetone glucose serves as starting material for the preparation of many partially substituted sugars. The details of its preparation have been repeatedly undergoing modification. Originally, ordinary α-glucose served for its preparation. The process, however, was very laborious and the yield was rather small for the reason that, prior to condensing with acetone, the sugar was converted into its methylacetal, as the α-glucose itself was supposedly too insoluble to react with acetone. Fischer therefore suggested the use of β-glucose, the more soluble form of this sugar, for condensation with acetone. The use of absolutely dry and alcohol-free acetone was also urged for the preparation of diacetone glucose.

Since we were in need of comparatively large quantities of the condensation product, we resolved to make a new attempt to prepare diacetone glucose from the α isomer. As will be seen from Table I diacetone glucose can be advantageously prepared from this isomer. It is true that the yield of diacetone glucose per unit weight of the employed sugar is at a maximum when the conditions of Fischer are observed; namely, when β-glucose and pure dry acetone are employed for the reaction. If, however, economy of time is considered, then per unit time, more product is obtained when diacetone is made from the common form of glucose even with ordinary commercial acetone, and surely when pure and dry acetone is employed. From the view of the cost of materials per unit weight of the diactone glucose obtained and from the view-point of purity of the resulting material, no one method offers any advantage over the other.

In the course of preparation of diacteone glucose, there always remains a residue. Fischer and Rund[1] mentioned this fact in describing the method of preparation from β-glucose. They stated that the residue consisted of the α isomer. In our experience, however, the residue consisted of the original form used for condensation. This is important to bear in mind when one is inclined to use the β form for the preparation of diacetone glucose, for the reason that the residue can be reemployed.

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[1] Fischer, E., and Rund, C., *Ber. chem. Ges.*, 1916, xlix, 93.

TABLE I.

Sugar in 100 gm. lots.	Acetone.	Residue.	Diacetone yield.	Average yield.
		gm.	*gm.*	*gm.*
β-glucose.	Anhydrous.	42	56	55
		42	55	
α-glucose.	"	60	39	36
		62	34	
β-glucose.	Ordinary.	49	45	42
		56	39	
α-glucose.	"	73	26	25
		68	24	

On one recrystallization from petroleum ether the substances by every process melted at 110°C.

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ON MONOACETONE BENZYLIDENE GLUCOSE.

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(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, July 9, 1923.)

In a previous publication,[1] it was reported that two different phosphoric esters of glucose were obtained depending upon the sugar derivative used for condensation with phosphorus oxychloride. In the substance obtained from diacetone glucose, the phosphoric acid radicle seemed to be attached to one of the carbon atoms other than 1 and 6. In the product obtained from monoacetone monobenzylidene glucose, the phosphoric acid radicle was more firmly attached to the sugar molecule and from this it could be concluded that it was linked to carbon atom 6. Two alternative explanations could be given to this observation. Either diacetone glucose and monoacetone benzylidene glucose have their free hydroxyl in a different position, or, in monoacetone benzylidene glucose, the hydroxyl in position 6 is set free before the benzaldehyde radicle is completely liberated (possibly the benzaldehyde radicle migrates from positions 5 and 6 to others). The structure of diacetone glucose has been recently explained through the work of Karrer and Hurwitz,[2] Levene and Meyer,[3] and Freudenberg and Brauns.[4] It was then necessary to correlate the structure of monoacetone benzylidene glucose with that of diacetone glucose. This was accomplished. It was found that the two derivatives gave the identical monoacetone benzoyl glucose. Also, the same monomethyl glucose was obtained from diacetone glucose and from monoacetone benzylidene glucose. The two samples of monomethyl glucose had the same optical rotations and the same melting points. On oxidation, the monomethyl glucose

[1] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1922, liii, 431.

[2] Karrer, P., and Hurwitz, O., *Helvetica Chim. Acta*, 1921, iv, 728.

[3] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1922, liv, 805.

[4] Freudenberg, K., and Brauns, F., *Ber. chem. Ges.*, 1922, lv, 3233.

from monoacetone benzylidene glucose gave a crystalline product as did the monomethyl glucose prepared from diacetone glucose. Unfortunately, the supply of monoacetone benzylidene glucose was very small. The substance is much more difficult to prepare than diacetone glucose and the yield of methylsaccharicanhydride was too small for analysis. However, the melting point was at 207°C., which is identical with that of 1.4-anhydro-3-methylsaccharic acid. Thus, the evidence adduced is sufficient to assume for monoacetone benzylidene glucose a structure analogous to that of diacetone glucose, and is to be regarded as 1,2-acetone-5,6-benzylidene glucose.

EXPERIMENTAL.

Monoacetone Benzylidene Glucose.—The method for preparing this substance as previously described by us was slightly modified.

50 gm. of monoacetone glucose were heated with 300 cc. of freshly distilled benzaldehyde and 100 gm. of anhydrous sodium sulfate in an oil bath at 170° for 5 hours. The reaction product was filtered from the sodium sulfate while still hot and concentrated under diminished pressure at 130°C. (oil bath temperature). The product was cooled and poured into 1,000 cc. of cold petroleum ether, 30–60°. A sticky mass which solidified on stirring settled out. This brownish mass was washed several times with cold petroleum ether and subsequently with a small quantity of ether, and then filtered. A practically colorless substance was obtained. It was recrystallized several times from 95 per cent alcohol and decolorized with norit. The petroleum ether solutions were concentrated and an additional yield of crude material was obtained in this manner. It was recrystallized from 95 per cent alcohol. The pure substance melted at 144° and analyzed as follows:

0.1052 gm. substance: 0.2408 gm. CO_2 and 0.0618 gm. H_2O.

$C_{16}H_{20}O_6$.	Calculated.	C 62.30, H 6.54.
	Found.	" 62.42, " 6.58.

Methyl Benzylidene Glucose and Its Hydrolysis to Methyl Glucose.—15 gm. of monoacetone benzylidene glucose were methylated with an excess of dimethyl sulfate and 30 per cent sodium hydroxide at 70°

(water bath temperature). The reaction product was extracted with chloroform and dried with anhydrous sodium sulfate. The chloroform was removed under diminished pressure. The syrup which remained was hydrolyzed.

For this purpose, it was dissolved in 75 cc. of 50 per cent alcohol, containing 0.4 per cent of hydrochloric acid, and heated in boiling water with reflux for 70 minutes. The solution was cooled and the hydrochloric acid removed with silver carbonate. The solution was then treated with hydrogen sulfide, filtered, decolorized with norit, and concentrated to a syrup under diminished pressure. The residue was taken up in absolute alcohol and again evaporated to dryness in order to remove traces of water. This operation was repeated several times. The syrup was then taken up in warm methyl alcohol and allowed to stand in a desiccator over sulfuric acid. On standing, crystals of methyl glucose separated. These had the following optical rotations in water.

Initial

$$[\alpha]_D^{20} = \frac{+1.03° \times 100}{1 \times 1} = +103°$$

Final.

$$[\alpha]_D^{20} = \frac{+0.57° \times 100}{1 \times 1} = 57°$$

The substance melted at 156–157°C.

Oxidation of Methyl Glucose.—0.450 gm. of the sugar was dissolved in 25 cc. of 50 per cent nitric acid, allowed to stand at room temperature for 42 hours, and then evaporated in a clock-glass on a boiling water bath, as described in a previous publication.[8] The residue was taken up in a mixture of ether and acetone. The solution was filtered and allowed to evaporate spontaneously. A small crystalline deposit formed. It was filtered and washed with a mixture of acetone and ether. The crystals darkened at 190° and melted at 207°C.

Monoacetone Benzoyl Glucose.—15 gm. of monoacetone benzylidene glucose were added to a solution of 15 cc. of pyridine and 7.5 of benzoyl chloride. The temperature rose slightly and all the sugar dissolved. No pyridine hydrochloride separated and the solution was kept at 45° for 24 hours. The product was then dissolved in chloroform and washed with very dilute sulfuric acid, dilute sodium bicarbonate, and water. The chloroform solution was dried with anhydrous sodium sulfate and concentrated under diminished pressure to a very small volume and poured into an excess of petroleum ether. A crystalline product was obtained which was recrystallized from methyl alcohol. This melted at 198°. Mixed with monoacetone benzoyl glucose obtained from diacetone glucose, it melted at the same temperature. The substance analyzed as follows:

0.1038 gm. substance: 0.2262 gm. CO_2 and 9.060 gm. H_2O.

$C_{16}H_{20}O_7$. Calculated. C 59.23, H 6.22.
Found. " 59.42, " 6.55.

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, September, 1923, Vol. lvii, No. 2, pp. 323–327.]

ON EPICHITOSAMINE PENTACETATE.

BY P. A. LEVENE.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, July 9, 1923.)

The configuration of carbon atom 2 in nitrogenous sugars and their corresponding sugar acids remains a matter of speculation. In a previous publication[1] some analogies between chitosaminic and mannonic acids and between chondrosaminic and talonic acids were pointed out. Thus, on the basis of these analogies, one could surmise that chitosamine had the configuration of 2-aminomannose and chondrosamine that of 2-aminotalose. Very recently however, Irvine and Earl[2] have pointed out that the difference between the molecular rotations of the α and β isomers of glucosamine was identical with the difference between the glucoses and therefore concluded that chitosamine had the configuration of glucosamine. It cannot be denied that the argument of Irvine and Earl may be correct. The possibility that chitosamine may have the configuration of 2-aminoglucose was never denied. The theory, however, is in need of further proof. The properties of glucose and of chitosamine which Irvine compares are those associated with the properties of carbon atom 1. In glucose and in chitosamine, carbon atom 1 functions regularly; in mannose, it functions irregularly. To this argument of Irvine and Earl may be added the fact that carbon atom 1 in epichitosamine hydrochloride, similarly to mannose, functions irregularly. Thus, on the basis of the conduct of carbon atom 1, one might be justified in grouping chitosamine with glucose and epichitosamine with mannose. However, it is not excluded that in the nitrogenous sugars carbon atom 1 functions differently than in ordinary sugars. Levene and

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[1] Levene, P. A., Hexosamines, their derivatives, and mucins and mucoids, Monograph of The Rockefeller Institute for Medical Research, No. 18, New York, 1922.

[2] Irvine, J. C., and Earl, J. C., *J. Chem. Soc.*, 1922, cxxi, 2376.

Meyer[8] have already described a peculiar behavior of this carbon atom in the epiglucosamine of Fischer, Bergmann, and Schotte. In the present paper, we wish to point out another unusual peculiarity. Both in a sugar and in a sugar acid, the direction of the rotation of carbon atom 2 may be determined either from the rotations of a pair of epimeric monocarboxylic sugar acids, or from the rotations of the α and β forms of the epimeric sugars. Taking glucose and mannose, it is possible to determine the rotation of carbon atom 2 from the rotations of the salts, amides, or phenylhydrazides of gluconic and mannonic acids. It is also possible to determine it from the values of the rotations of the α and β forms of glucose and mannose. Designating in each sugar the group of carbon atom 1 by A, that of carbon atom 2 by B, and the remaining part of the molecule by C, one may represent the molecular rotation of glucose by the equation $A+B+C=m$ and that of mannose by $A_1-B+C=n$. When the molecular rotations of the sugars and of the carbon atoms 1 are known, the directions and the values of B can be readily determined from the equations $(A+B+C)-(A_1-B+C)=m-n$ and $(A+B+C)+(A_1-B+C)=m+n$. In the system glucose and mannose either one of the two methods gives the direction to the right for carbon atom 2 of glucose and to the left for carbon atom 2 of mannose. The same result is obtained when, instead of glucose and mannose, the pentacetates are used for calculation.

In the system chitosamine and epichitosamine one encounters a deviation from the rule. From the system chitosaminic and epichitosaminic acids carbon atom 2 is found to rotate to the left in chitosaminic and to the right in epichitosaminic acid. When the direction of the rotation of carbon atom 2 is determined from the values of the molecular rotations of chitosamine and epichitosamine, it is found that carbon atom 2 rotates to the left in epichitosamine and to the right in chitosamine. It is hardly probable that in the process of oxidation of a sugar to the corresponding monocarboxylic acid, the configuration of carbon atom 2 undergoes a change. Since a sugar and its monocarboxylic acid differ only by the character of carbon atom 1, it is more logical to assume that the differences in the direction of the rotations of the two carbon atoms 2 are brought about by differences in the structure of the carbon atoms 1. The condition is somewhat analogous to that of *l*-aspartic acid and its ester. The acid rotates to the right, whereas the ester rotates to the left. Thus, the task of settling the problem of allocation of the amino group in nitrogenous sugars is becoming rather more complicated than simplified. One is tempted to accept for chitosamine the configuration of 2-aminoglucose and for epichitosamine that of 2-aminomannose, inasmuch as the α and β isomers of chitosamine and glucose behave normally, and the corresponding forms of epichitosamine and mannose have an abnormal behavior. However, if this conclusion were accepted the case of epichitose would be puzzling. Epichitose, which has the structure of 2,5-anhydroglucose, resembles epichitosamine in regard to the function of its carbon atom 1. Both these sugars show no mutarotation and both possess levo-rotation. Thus, if one takes glucose for a standard of comparison, one may arrive with Irvine and Earl at the conclusion that chitosamine has the structure of 2-aminoglucose. On the other hand, taking 2,5 anhydroglucose as the standard, the conclusion may be reached that epichitosamine has the structure of 2-aminoglucose.

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[8] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1923, lv, 221.

In the present communication, data are given for the determination of the direction of the rotation of carbon atom 2 in chitosamine and in epichitosamine. Chitosamine hydrochloride was fractionated by Irvine and Earl into the α and β isomers. Epichitosamine showed no mutarotation, and its specific rotation was very low. Because of these properties, it was uncertain whether epichitosamine had the glucosidic or the ordinary aldehydic structure. On the other hand, since the substance was levo-rotatory, it was thought that it might consist primarily of the β form, if it had the glucosidic structure. In view of the scarcity of the material, it was thought advantageous to prepare the pentacetate.

Regarding the pentacetates, experience has shown that in the *d* series, the β isomers are transformed into the α forms to the extent of not less than 90 per cent by heating in acetic anhydride containing zinc chloride. In the *l* series, the reaction is reversed. β-Epichitosamine pentacetate was prepared and was found to have a molecular rotation of $-3{,}890°$ in acetic anhydride. This form is to be regarded as the β isomer. The α form had a molecular rotation of not less than +19,450°. Hence, the difference of the rotations of the two isomers was not less than +23,340°.

From the equation $[A + (\pm B) + C] - [A_1 + (\mp B) + C] = m - n$ (substituting for A its value + 17,965, for A_1 the value − 12,643, for m its value 36,400, and for n the value −3,890) it follows that $B = \pm 4{,}841$; from the sum of factors of the same equation $C = + 13{,}594$. Substituting these values, respectively, in equation $A + (\pm B) + C = 36{,}400$ and in equation $A_1 + (\mp B) + C = -3{,}890$, a positive value is obtained for B (carbon atom 2) of chitosamine and a negative value for epichitosamine.

EXPERIMENTAL PART.

Several lots of epichitosamine hydrochloride, each of 5.0 gm., were taken up in 27.0 cc. of acetic anhydride and 27.0 cc. of pyridine and allowed to stand 24 hours at 37°C. and 24 hours at room temperature. The reaction product was transferred into a mixture of ice and water and the pentacetate shaken out with chloroform. The chloroform solution was washed with water, dilute mineral acid, then with a cold solution of sodium bicarbonate acid, and finally with water. The chloroform solution was dried over anhydrous sodium sulfate and concentrated to dryness. The residue was dissolved in alcohol and evaporated to dryness. This operation was repeated several times. The final residue was crystallized from alcohol. For analysis, it was recrystallized from alcohol. The long white needles melted at 158–159°C. (corrected) and analyzed as follows:

0.1989 gm. substance: (Kjeldahl) 5.25 cc. 0.1 N acid.
0.1068 " " : 0.1934 gm. CO_2 and 0.0600 gm. H_2O.

$C_6H_8NO_5(CH_3CO)_5$. Calculated. C 49.49, H 5.96, N 3 60.
Found. " 49.38, " 6.28, " 3.69.

The rotation of the substance in chloroform was:

$$[\alpha]_D^{28} = \frac{-0.54^\circ \times 100}{1 \times 3} = -18^\circ$$

Conversion of the β into the α Isomer.—A 3 per cent solution of the pentacetate in acetic anhydride, containing 1 per cent of zinc chloride, was kept in a thermostat at 40°C. and the rotation of the solution measured at intervals. The initial rotation was −0.30° in a 100 mm. tube. The solution reached an equilibrium after 120 hours and then had a rotation of +1.45°. In a second experiment, a 3 per cent solution of the substance was made in acetic anhydride, containing 3 per cent of zinc chloride, and the solution was refluxed on a boiling water bath. The initial rotation was +0.30° in a 100 mm. tube, and after 20 minutes the rotation was +1.50°. It was not possible to make further readings as the solution darkened.

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PREPARATION OF α-MANNOSE.

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Mannose has been prepared only in the form of its β isomer with a specific rotation of −17°. The specific rotation of the α form was obtained indirectly by Hudson and Yanovsky[1] and was found to be +34 or +35°. The difference of the specific rotations was, therefore, +52°, whereas in the case of the two glucoses, it is +94°. The value for the two mannoses is exceptional. A value of the same magnitude was observed in the case of only two other sugars; namely, lyxose and rhamnose. Mannose occupies an exceptional position in other respects. The ratio of the initial and final solubilities of β-mannose in 80 per cent ethyl alcohol and in methyl alcohol is 1:5 and 1:5.6, respectively, and in that of the glucoses and of the galactoses, it is approximately 1:2. Also, it is peculiar that the difference of the specific rotations of two mannoses is equal to only 55 per cent of that of the two glucoses, whereas in the case of the pentacetates, the value of the difference is 80 per cent of that of the glucoses. In fact, the difference of the molecular rotations of the pentacetates of the mannoses (31,000) is very near to that of the galactoses (32,700). Because of the abnormal behavior of mannose and because this behavior has been made the basis of a theory of the structure of chitosamine,[2] it seemed desirable to prepare the unknown isomer of mannose. This has now been accomplished.

In the process of its preparation, another abnormality of mannose came to light. Under conditions when glucose and galactose appear in the β form, mannose crystallizes in the α form and *vice versa*. For glucose there exist two convenient methods for the preparation of the β isomer. The one is that of Behrend,[3] which consists in crystallizing glucose from a pyridine solution, the other is that of Hudson and Dale,[4] and consists in crystallizing glucose from hot glacial acetic acid and alcohol. A third much simpler and more economical method was discovered in the course of this work; namely, crystallization from aqueous ammonia and alcohol. Both glucose and galactose crystallize under this simple condition in the β form. Under all these three conditions, mannose crystallizes in the α form. This observation suggested the possibility that the configuration of the carbon atom 1 in β-glucose was the same as in α-mannose; namely, that the hydroxyl in both was to the right. Against such an assumption militates the behavior of their pentacetates. It is known that pentacetates of sugars may be transformed into the isomeric forms by warming them in a solution of acetic anhydride containing zinc chloride. A scrutiny of all the known instances of transformation of pentacetates reveals the fact that in the *d* series, the β isomers are transformed into the α isomers, to the extent of over 90 per cent, whereas the transformation of the α form is minimal. In other words, in a solution of acetic anhydride and zinc chloride, the equilibrium mixture consists almost entirely of the α isomer. In the *l* series, the equilibrium is reversed. In this respect, the conduct of mannose conforms to all other known sugars. Hence, one may assume that the position of the hydroxyl on the carbon atom 1 in α- and β-mannose is the same as in the corresponding form of other simple sugars.

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[1] Hudson, C. S., and Yanovsky, E., *J. Am. Chem. Soc.*, 1917, xxxix, 1013.

[2] Irvine, J. C., and Earl, J. C., *J. Chem. Soc.*, 1922, cxxi, 2376.

The specific rotation for α-mannose was found + 30° in water and +35° in 80 per cent alcohol. This value is in complete agreement with the value calculated by Hudson and Yanovsky. Furthermore, the possession of the two forms permitted the establishment of the fact that the form with the specific rotation of −17° was free from the α form, and the mannose with a specific rotation +35° was free from the β form. This conclusion was reached on the comparison of the solubilities of the α and β isomers separately and of a mixture of the two isomers. The solubility of the mixture was equal to the sum of the solubilities of each of the isomers separately. Furthermore, from the initial and final solubilities of the α isomer, the specific rotation of the β form is calculated $-16.5°$. Hudson and Yanovsky find for the same form in 80 per cent alcohol $-14.9°$. The rotation of our pure α form was slightly higher. The differences are, however, within the limits of possible error. The evidence thus far adduced supports the view that the mannose with the specific rotation of $+35°$ is the pure α isomer. However, mannose with the specific rotation of $+35°$ may still contain a third isomer and yet on the basis of its initial and final solubility, it may give the correct value for the specific rotation of the β isomer and also give a combined solubility of the two forms as if they were pure α and β isomers.

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[3] Behrend, R., *Ann. Chem.*, 1907, cccliii, 106.

[4] Hudson, C. S., and Dale, J. K., *J. Am. Chem. Soc.*, 1917, **xxxix**, 325.

A sufficient proof of the purity of each form should be based on the following considerations. It was shown independently by Hudson[5] and by Lowry[6] that mutarotation is a balanced reaction which follows the monomolecular form of the mass law and can be expressed by the equation

$$k_1 + k_2 = \frac{1}{t} \log \frac{r_\infty - r_0}{r_\infty - r_t}$$

and, furthermore, that the rate of transformation of each form into the other can be measured from the rate of their maximum solubilities by the equation k_1 (when the α form is considered) or k_2 (when the β form is under consideration) $= \frac{1}{t} \log \frac{S_\infty - S_0}{S_\infty - S_t}$ (S stands for solubility). On the basis of these equations, it is possible, on the one hand, to determine the sum of $k_1 + k_2$ (from the mutarotation of either one of the two isomers) and, on the other hand, k_1 and k_2 independently (from the rate of solubilities of each). Hence, the sum of k_1 and k_2 obtained independently should be equal to $k_1 + k_2$ obtained on the basis of mutarotation. It was intended to apply this test for the purpose of establishing the purity of the mannose with the specific rotation of $+35°$ (in 80 per cent alcohol). It was surprising, however, to find that the value of $(k_1 + k_2)$ measured on the α form differed from that measured on the β form. The measurements were made in 80 per cent alcohol at several temperatures. In order to control the accuracy of our measurements, the mutarotations of α- and β-glucoses in solutions of 60 and 80 per cent ethyl alcohol were measured and found identical within the limits of error. Taking as a standard of comparison the coefficient at 12.5°C. it was of the order of magnitude of 0.00150 for the β form (recalculated for 20°C. it is 0.00333; this is in close agreement with the value of 0.00363 found by Hudson and Yanovsky), whereas the isomer gave the value for $k_1 + k_2 = 0.00240$ (recalculated for 20°C. = 0.00533). One must add, however, that the entire range of change of the α form from the initial to final is rather small, so that only few measurements can be relied upon for calculating the velocity coefficient. However, since the difference between the two coefficients was constant, it may be regarded as real. It is important to compare their velocity coefficients in water. These, however, can be measured only at lower temperatures inasmuch as the α-mannose solution reaches equilibrium very rapidly. In view of the atmospheric humidity during the spring and summer months, this experiment has to be postponed until the winter. For the present, therefore, the possibility is not excluded that the mannose with a specific rotation of +35° (in a solution of 80 per cent alcohol) contains in addition to the α form still a third form.

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[5] Hudson, C. S., *J. Am. Chem. Soc.*, 1904, xxvi, 1065.

[6] Lowry, T. M., *J. Chem. Soc.*, 1904, lxxxv, 1551.

This assumption may explain the fact that in the equilibrium mixture in 80 per cent alcohol and in methyl alcohol, the β form of mannose comprises only 18 per cent of the total, whereas in the cases of glucose and galactose, it comprises practically 50 per cent. It would also explain the fact that the value of the difference of the molecular rotations of the α- and β-mannoses is only 40 per cent of the normal value, whereas in the case of pentacetates, the value is 80 per cent of the normal.

SUMMARY.

1. A form of mannose was prepared which had a specific rotation of +30° in water and +35° in 80 per cent alcohol.
2. This form has the specific rotation calculated for α-mannose.
3. The conditions which lead to the formation of α-mannose are the same which lead to the formation of β-glucose and β-galactose.

4. The configuration of the carbon atom 1 in the α and β isomers, respectively, in glucose, galactose, and mannose is identical.

5. It is possible that the mannose with the specific rotation of +35° contains besides the α isomer still a third form.

6. A convenient and economical method is given for the preparation of β-glucose and of β-galactose.

EXPERIMENTAL PART.

Preparation of α-Mannose by the Pyridine Process.—25.0 gm. of α-mannose ($[\alpha]_D^{20} = -12°$) were heated with 30 cc. of pyridine until solution was completed. To the pyridine solution 250 cc. of 98.5 per cent ethyl alcohol were added and the solution was allowed to stand at room temperature for about 3 hours. The solution turned into a solid mass. The first yield of α-mannose was about 60 per cent. The mother liquors were generally concentrated under diminished pressure and taken up again in hot pyridine. To the solution 98.5 per cent alcohol was added. On repeating the operation, α-mannose can be converted into the α isomer practically without loss. The rotation of the sample reported in this experiment in aqueous solution was

$$[\alpha]_D^{20} = \frac{+0.90° \times 100}{1 \times 3} = +30°$$

In some experiments, the material had a specific rotation of $[\alpha]_D^{0} = +28°$.

Preparation of α-Mannose by the Glacial Acetic Acid Process.— 50.0 gm. of α-mannose ($[\alpha]_D^{20} = -11°$) were dissolved in 10 cc. of hot water. 100 cc. of hot glacial acetic acid were added to the solution. On standing, the solution turned into a solid mass. The yield was 25.0 gm. and the specific rotation was

$$[\alpha]_D^{20} = \frac{+0.75° \times 100}{1 \times 3} = +25°$$

Also in these experiments, the mannose remaining in the mother liquors was easily recovered.

Preparation of α-Mannose by the Ammonia Process.—10 gm. of β-mannose were dissolved in 5 cc. of concentrated aqueous ammonia.

To the solution, 100 cc. of absolute alcohol and then ether were added as long as an oil continued to settle out. The oil was taken up in small portions of dry methyl alcohol until it crystallized. The dry substance had the following specific rotation.

$$[\alpha]_D^{20} = \frac{+0.72° \times 100}{1 \times 3} = +24°$$

Also, in this process the mannose remaining in the mother liquor could be completely recovered.

Purification of α-Mannose.—For purification, the crude material was allowed to stand for 24 hours in double its weight of 80 per cent alcohol. The residue was shaken for 5 minutes with a small portion of 80 per cent alcohol at 20°C. To a portion of the filtrate a few drops of ammonia were added and the optical rotation was measured. The extraction was continued until the rotation remained constant for three subsequent extractions. The rotation of these extracts in a 1 dm. tube was $\alpha = +2.30°$. The residue was then washed with absolute alcohol and ether and dried under diminished pressure at 50°C. The substance melted into a semisolid mass at 133°C. (corrected) and turned liquid and decomposed at 205°C. It had a specific rotation in water of $[\alpha]_D^{20} = +30°$, and in 80 per cent alcohol of +35°.

Initial and Final Solubilities of α-Mannose in 80 Per Cent Alcohol.—The experiment was carried out at 20°C. 10.0 gm. of the dry α form were shaken in a glass stoppered flask for 5 minutes with 40 cc. of the solvent. About 10 cc. of the supernatant liquid were withdrawn by a Lowry pipette, 2 drops of ammonia water were added to the solution, and the rotation was measured. $[\alpha]_D$ was +2.30°. The flask with the remainder of the suspension was allowed to stand for 24 hours when another sample of 10 per cent was withdrawn. The rotation was +2.80°.

The rotation of the β form is calculated from the equation $0.821 (35) + 0.179 (x) = 25.7$. 25.7 is the equilibrium specific rotation of mannose in 80 per cent alcohol. x (the rotation of the β form) is +16.5°.

Initial and Final Solubilities of β-Mannose in 80 Per Cent Alcohol.—The experiment was carried out in the same manner as in the case of α-mannose. 10.0 gm. of β-mannose were suspended in 75 cc. of 80 per cent alcohol. The rotation of the first extract was +0.38°, of the final +1.80°. The specific rotation of the α form, calculated from the equation $0.188\ (-16.5) + 0.812x = 25.7$, is + 35°.

The Solubility of a Mixture of α- and β-Mannoses.—The β-mannose was purified in the same way as the α isomer. It melted into a semisolid mass at 140°C. (corrected), and then behaved as the α form. A concentrated solution in 80 per cent alcohol in equilibrium gave an optical rotation of +0.38° in a 100 mm. tube.

A mixture of 3 gm. of β-mannose and of 7.0 gm. of α-mannose was shaken for 5 minutes at 20°C. with 30 cc. of 80 per cent alcohol. The filtrate, after addition of a drop of ammonia, had the optical rotation of $[\alpha]_D^{20} = +2.67°$ in a 100 mm. tube. The calculated value is + 2.68°.

Mutarotation of α- and β-Mannoses at 25°C.—5 gm of dry α-mannose were shaken in a thermostatic bath at 25°C. for 5 minutes. The supernatant liquor was rapidly filtered. 3 gm. of the β form were treated in the same way. The measurements were taken in tubes provided with jackets through which circulated the water from the same thermostatic bath. The changes of rotation were as follows:

Time.	α Form	$k_1 + k_2$	Time	β Form.	$k_1 + k_2$
min.			*min.*		
0	+6.95		0	−0.72	
27	+6.25	0.00775	30	−0.07	0.00545
57	+5.79	0.00795	62	+0.43	0.00572
89	+5.50	0.00770	91	+0.73	0.00580
116	+5.31	0.00850	120	+0.95	0.00602
Equilibrium.	+5.12	Average. . .0.00792	Equilibrium.	+1.34	Average. . . .0.00549

Mutarotation of α- and β-Mannoses at 12.5°C.—These measurements were carried out in a room in which the temperature was kept constant at 12.5°C. 0.500 gm. of dry β-mannose was dissolved in 25 cc. of 80 per cent alcohol. It took about 2½ minutes to complete solution. 3 gm. of α-mannose were shaken 5 minutes with 3 cc. of 80 per cent alcohol. Under these conditions, the concentrations of the α- and β-mannoses were approximately the same.

The course of the mutarotation was as follows:

Time.	α Form.	$k_1 + k_2$	Time.	β Form.	$k_1 + k_2$
min.			*min.*		
0	+1.33		0	−0.66	
34	+1.26	0.00219	30	−0.57	0.00169
76	+1.19	0.00239	59	−0.37	0.00144
113	+1.14	0.00247	97	−0.22	0.00141
147	+1.11	0.00255	121	−0.11	0.00148
Equilibrium.	+0.95	Average. . .0.00240	Equilibrium.	+0.97	Average.. .0.00150

Two other experiments were performed. The respective values were 0.00241 and 0.00239 for the α, and 0.00170 and 0.00141 for the β form. Recalculated for 25°C. by the coefficient of Hudson and Sawyer (2.6 for 10°) the values given in the table become 0.00753 for the α form and 0.00567 for the β form. These values agree very closely with those found in the experiments reported above.

Preparation of β-Glucose.—200 gm. of α-glucose were dissolved in about 50 cc. of hot water; to this solution, 20 cc. of concentrated ammonia water were added. The solution was allowed to stand 5 minutes. Then 200 cc. of 98.5 per cent alcohol were added. To the resulting solution ether was added as long as an oil settled out. The supernatant liquid was then decanted and to the syrup methyl alcohol was added in small portions, while stirring and scratching on the walls of the vessel were continued. The glucose crystallized immediately. The yield was 140 gm. The specific rotation of the crude material was $[\alpha]^{20}_{D} = +23°$. The material remaining in the mother liquor was easily recovered.

Preparation of β-Galactose.—50 gm. of α-galactose were dissolved in 40 cc. of concentrated ammonia water in the cold. 200 cc. of cold alcohol were added and then ether was added as long as oil settled out. The supernatant liquid was decanted, and to the oil small portions of methyl alcohol were added, while stirring and scratching on the walls of the vessel were continued. The crystallization began almost at once. The specific rotation of the crude β form was $[\alpha]^{20}_{D} = +63°$.

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THE TWO ISOMERIC CHONDROSAMINE HYDROCHLORIDES AND THE RATES OF THEIR MUTAROTATION.

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The first sample of chondrosamine hydrochloride isolated by Levene and La Forge[1] had an initial rotation of $[\alpha]_D^{20} = +129°$, which reached an equilibrium at the rotation of $[\alpha]_D^{20} = +95°$. All subsequent preparations, both synthetic and natural, had an initial specific rotation in the neighborhood of $[\alpha]_D^{20} = +51–60°$ and an equilibrium rotation of $[\alpha]_D^{20} = +95°$. Since chondrosamine belongs to the *d* series, the second form is to be regarded as the β isomer. In this respect, chondrosamine represents one of the few sugars which under normal conditions, crystallizes in the β form. The common form of most other simple sugars, with the exception of mannose, is the α form.

The knowledge of the rotation of the α and β isomers is important for the purpose of obtaining information as to the value and the direction of the rotation of carbon atom 2 in the sugars. The bearing of the direction of carbon atom 2 on the configuration of sugars and sugar acids has been discussed in the paper on epiglucosamine pentacetate.[2] In view of this, an effort was made to prepare both isomers of chondrosamine hydrochloride in pure form. Since the prevailing material consists of the β isomer, the first step was to obtain the equilibrium form. A convenient way to prepare this form is given in the experimental part. This material was extracted repeatedly either with methyl alcohol or with a solution consisting of equal parts of absolute ethyl and methyl alcohols. By the addition of ether to the filtrate, a substance was obtained which had a higher rotation than the initial substance. On repeating the operation, the rotation of the more soluble form gradually increased and finally reached the value $[\alpha]_D^{20} = +121°$.

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[1] Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, 1914, xviii, 127.

[2] Levene, P. A., and Meyer, G M., *J. Biol. Chem.*, 1923, lv, 221.

On the other hand, a method was found to purify the β isomer so that its rotation came down to $[\alpha]_D^{20} = +47°$. The extrapolated initial rotation was found to be $[\alpha]_D^{20} = +44.5°$. Thus, the difference of the molecular rotation of the two forms is 16,485. It is possible that the α form was not yet absolutely pure and that the pure substance possesses a slightly higher rotation than + 121°, but the difference of the molecular rotations of the two forms is practically identical with the normal value for the majority of sugars. It is also very close to the value obtained by Irvine and Earl for the difference of the rotation of the two isomeric chitosamine hydrochlorides. The fact that the mutarotations of the α and β forms gave the same value for $k_1 + k_2$ speaks in favor of the purity of the two substances. The discrepancy in the rotation of the sample of Levene and La Forge and of the present samples may in part be due to the difference in the details of the technique of measurement. Whereas, in previous work the measurement was carried out in closed tubes, the present measurements were made in tubulated tubes.

EXPERIMENTAL PART.

Purification of Chondrosamine Hydrochloride.—3.0 gm. of crude material with a specific rotation of $[\alpha]_D^{20} = +80°$ were dissolved with heat in 80 cc. of concentrated hydrochloric acid. An additional 7.0 gm. of substance was added to the solution in small portions. To this solution alcohol was added until a crystalline deposit formed. The precipitate was filtered off while the mixture was still warm. This substance melted with decomposition at 187°C. 3 minutes after the substance was mixed with the solvent the rotation was

$$[\alpha]_D^{25} = \frac{+0.96° \times 100}{1 \times 2} = +48°$$

The mother liquors of this material were concentrated nearly to dryness and dissolved in a minimum amount of hot concentrated hydrochloric acid. Absolute alcohol was added until the substance began to crystallize. The optical rotation of this substance 4 minutes after solution was

$$[\alpha]_D^{25} = \frac{+1.38° \times 100}{1 \times 3} = +46°$$

The rate of mutarotation of the β form was as follows:

Time.	α	$k_1 + k_2 = \frac{1}{t}\log\frac{r_\infty - r_0}{r_\infty - r_t}$
4 min.	+0.96	
15 "	+1.20	0.0154
27 "	+1.40	0.0131
37 "	+1.49	0.0121
47 "	+1.57	0.0121
133 "	+1.84	Average.............0.0131
24 hrs.	+1.84	

On the basis of these values the extrapolated initial specific rotation was $[\alpha]_D^{20} = +44.5°$.

Preparation of α-Chondrosamine Hydrochloride.—50 gm. of the substance with specific rotation $[\alpha]_D^{15} = +0.56°$ were dissolved in 600 cc. of warm 60 per cent alcohol. The solution was allowed to remain on the water bath for half an hour when 600 cc. of acetone were added. An oily sediment formed. Methyl alcohol was then added until the sediment acquired a crystalline character and then 400 cc. of acetone were gradually added with stirring. The mixture was allowed to stand for 1 hour and then filtered. The molecular rotation of this material was $[\alpha]_D^{25} = +67.5°$. The filtrate was concentrated to a syrup under diminished pressure at 40°C. (water bath temperature). The syrup was dissolved in a little methyl alcohol. To the solution acetone was gradually added as long as its addition produced a cloudiness. The crystalline precipitate was filtered off after 15 minutes. Its specific rotation was $[\alpha]_D^{20} = +95°$. This value is that of the equilibrium form.

The pure α isomer was prepared by extracting the equilibrium form with methyl alcohol or with a mixture of ethyl and methyl alcohol. 20 gm. of the dry material were extracted at 15°C. for 3 minutes with 150 cc. of dry methyl alcohol and filtered. To the filtrate ether was added as long as it produced a turbidity. The crystalline precipitate was filtered off immediately. The extraction was repeated twice and the three crystalline deposits, obtained from the filtrates by the addition of ether, were combined. The yield was 5.0 gm. and the substance had the specific rotation of $[\alpha]_D^{20} = +121°$

On further purification, the rotation of the substance did not change. The final substance had a melting point of 185°C. and analyzed as follows:

0.1974 gm. substance: (Kjeldahl) 9.30 cc. 0.1 N acid.
0.1974 " " : (Volhard) 9.10 " 0.1 " $AgNO_3$.
0.1072 " " : 0.1306 gm. CO_2 and 0.0630 gm. H_2O.

$C_6H_{13}NO_5HCl$. Calculated. C 33.40, H 6.54, N 6.54, Cl 16.45.
Found. " 33.22, " 6.59, " 6.59, " 16.34.

The rotation of the substance was

$$[\alpha]_D^{20} = \frac{+2.42° \times 100}{1 \times 2} = +121°$$

In other experiments in which the mixture of the α and β forms had a specific rotation lower than +90°, the α isomer was obtained more successfully by extractions with a mixture of methyl and ethyl alcohol.

The rate of mutarotation of the substance was as follows. 7 minutes were required to dissolve the substance, filter the solution, and take the first reading:

Time.	α	$k_1 + k_2 = \frac{1}{t} \log \frac{r_\infty - r_0}{r_\infty - r_t}$
min.		
7	+2.36	
11	+2.21	0.0155
31	+2.07	0.0133
59	+1.96	0.0139
76	+1.90	0.0134
Equilibrium.	+1.86	Average. 0.0140

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ON THE IDENTITY OR NON-IDENTITY OF ANTINEURITIC AND WATER-SOLUBLE B VITAMINS.

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The problem to be reported in this paper has been the subject of much discussion in recent literature. A thorough analysis of the evidence on both sides of the question was presented first by Mitchel and later by Sherman and Smith. The very impartial scrutiny of all the evidence leads these authors to the conclusion that the evidence to date does not permit of a final decision in either direction. The present communication was made with a view of presenting evidence on the non-identity of the two vitamins. The observations were incidental in an effort to organize the work on the chemical nature of the water-soluble B vitamin. The original aim was to select a convenient source of vitamin B and a convenient laboratory animal. As the source of vitamin, various samples of yeast were tested and as laboratory animals, the pigeon and the white rat (descendants of Osborne and Mendel's colony, obtained through the courtesy of Prof. H. Sherman) were selected. It was then incidentally observed that certain samples of yeast which were sufficiently active to maintain normal growth in rats failed to protect pigeons fed on polished rice from polyneuritis. This observation led to a more careful investigation into the present problem. Three samples of bakers' yeast supplied by the Fleischmann Company, and one sample of brewers' yeast were employed in the investigation.

The results are summarized in the tables and chart, from which it is seen that the water-soluble B vitamin of four samples of yeast varied comparatively little. In daily doses of 0.200 gm., all the samples were effective. In 0.100 gm. doses, brewers' yeast and bakers' yeast No. 3 were sufficient to maintain normal growth, whereas Nos. 2 and 9 in such doses gave less satisfactory results.

Thus, the proportional difference between the best and the poorest samples was not greater than 2 to 1.

The results of feeding pigeons with the same samples of yeast were the following.

Brewers' yeast protected pigeons from polyneuritis in doses of 0.500 gm. per day; bakers' yeast No. 3 gave irregular results in doses of 0.200 gm. per day and protected completely in doses of 0.500 gm. per day. Bakers' yeast No. 9 failed to protect in doses of 0.500 gm. and gave irregular results in doses of 1.0 gm. (of three pigeons, one lived 50 days and two remained in perfectly normal condition after 70 days). Bakers' yeast No. 2 failed to protect even in doses of 2.0 gm. per day. Thus, No. 2 failed to protect pigeons from polyneuritis in doses at least four times as large as the protective dose of No. 3.

Occasionally, in a few earlier experiments, it seemed that an extract of a certain sample of yeast contained the protective power when the untreated yeast failed to do so. The extract was prepared following the directions of Osborne and Wakeman. A more careful investigation, however, revealed that the impression was erroneous. The Osborne and Wakeman fraction II obtained from yeast No. 2 was inactive in doses of 0.500 gm., whereas a similar fraction from the brewers' yeast carried a certain degree of protection in doses of 0.075 gm. per day and completely protected in doses of 0.125 gm.

It is noteworthy that, whereas the concentration of the protective principle was higher in yeast No. 3 than in the brewers' yeast, the concentrations of the principle in the Osborne and Wakeman fraction II were reversed, being lower in that from yeast No. 3 than from the brewers' yeast. All samples of the Osborne and Wakeman fraction II in daily doses of 0.020 to 0.050 gm. were effective in maintaining the normal growth of rats fed on the basal diet of Osborne and Mendel.

All postmortem examinations were made by Dr. P. Olitsky. Only animals sacrificed before death or found dead during laboratory hours were autopsied.

Thus, the material presented in this report contains additional evidence in support of the view that the antineuritic and the growth-promoting principle are not identical. However, it must be borne in mind that the final solution of the problem will be furnished only by the knowledge of the chemical nature of the active principles.

EXPERIMENTAL.

In the rat experiments, the technique of Osborne and Mendel was followed throughout. Young rats were placed in individual cages on the basal vitamin B-free diet until they had definitely lost weight, when vitamin in tablet form was given. Careful records of food consumption were kept and since the cages had wire mesh bottoms, there was little or no contamination or eating of feces.[1] All the tests were made on a series of four or more rats. The curves published are typical of the series.

The vitamin B-free basal diet employed was

	per cent
Casein	18
Salt mixture IV	4
Starch	54
Butter fat	9
Lard	13
Cod liver oil	2

For the pigeon experiments, birds weighing over 300 gm. were used. They were kept in individual cages and were forcibly fed 20 gm. of ground white rice, 18 cc. of distilled water, and the vitamin daily. All experiments were prophylactic and were continued at least 63 days on a series of three birds. Controls on 20 gm. of ground mixed grains were found healthy and normal.

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[1] Our cages were made of galvanized iron, 9 inches in diameter. They were made in three pieces, the wire mesh base with a rim to hold the cage, and a cover weighted with a lead rim. The cage stood in a deep agate pan over a layer of sand, which may be moistened with 2 per cent sulfuric acid in order to remove effectively any odor.

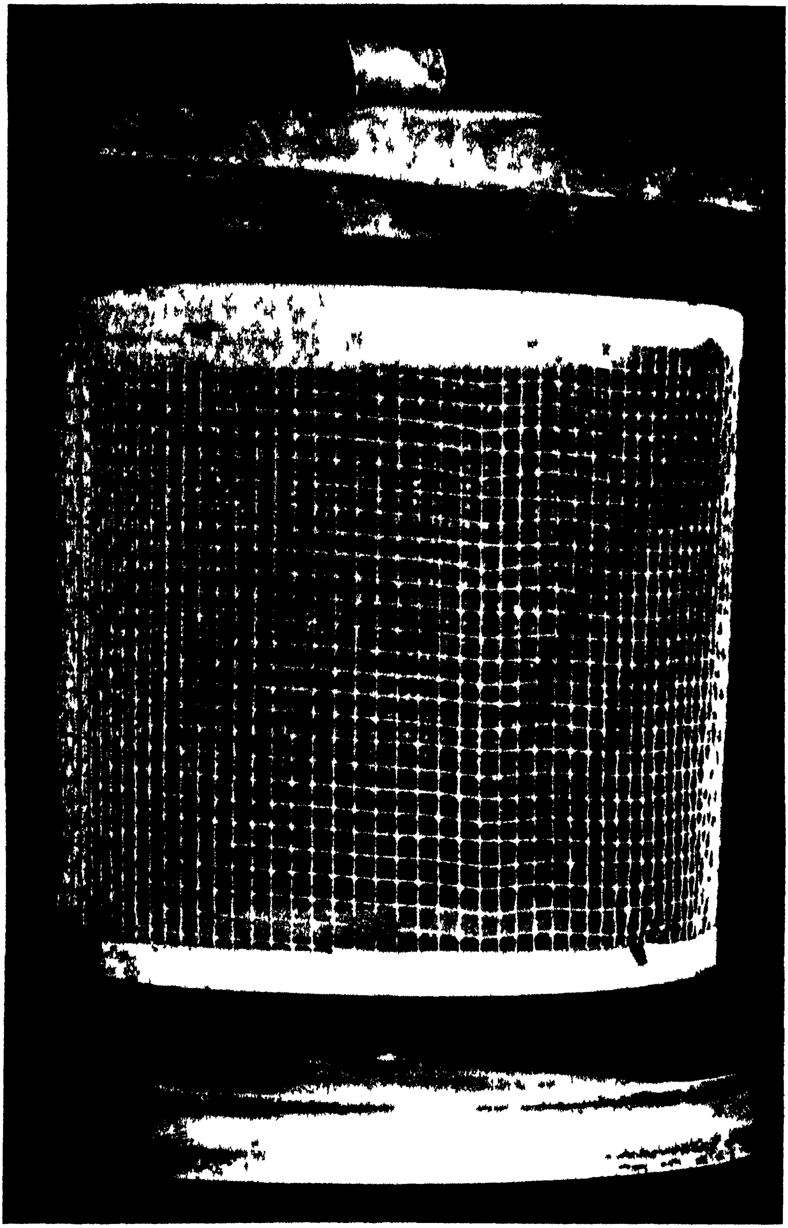

FIG. 1

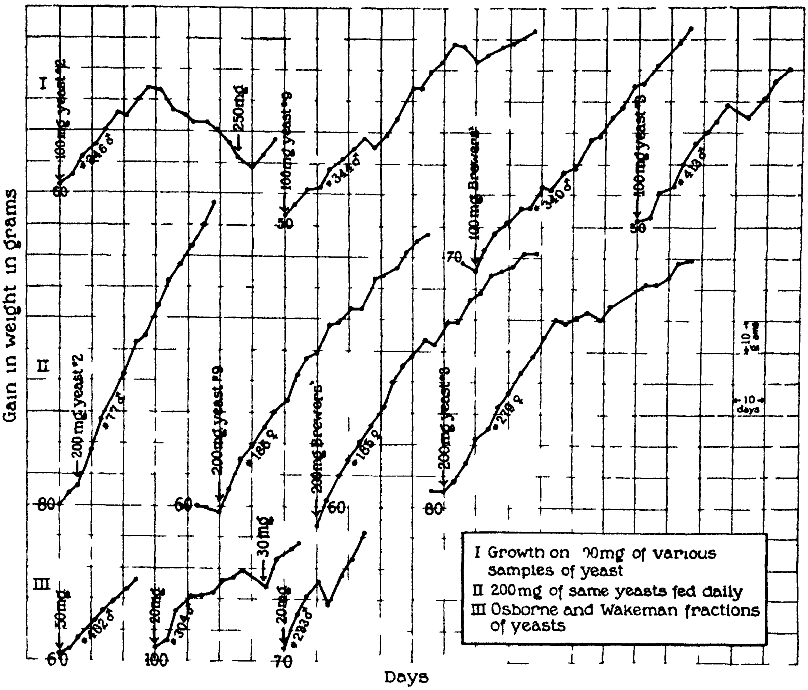

CHART 1

TABLE 1

Bakers' Yeast No 2 on Pigeons

Amount	Bird No	Length of life	Change in weight	Remarks
		days	*gm*	
200 mg	17	32	+2	Spastic killed
	18	20	+4	Died
	19	22	−32	Spastic, died
	20	20	0	" "
500 mg	74	21	−50	Convulsions
	75	14	−7	"
	76	17	+10	Crop full, spastic
1 gm	92	17	Crop full	Convulsions Autopsy, polyneuritis
	93	33	−106	Died
	94	30	+70	"
2 gm	177	77	−10	Autopsy, normal
	179	77		
	180	68	−55	Autopsy, polyneuritis

TABLE II.

Osborne and Wakeman's Fraction II from Bakers' Yeast No. 2.

Amount.	Bird No.	Length of life.	Change in weight.	Remarks.
mg.		*days*	*gm.*	
75	148	21	−35	Autopsy; polyneuritis.
	149	25	Crop full.	" "
	150	18	−27	Spastic.
125	151	28	−77	Autopsy; polyneuritis
	152	28	−35	Died
	153	21	−43	Autopsy; polyneuritis
150	181	21	−10	Autopsy; almost normal.
	182	26	−41	" polyneuritis
	183	26	Crop full.	Paralysis. Autopsy; polyneuritis.
175	154	27	−44	Spastic.
	155	28	Crop full.	Died.
	156	21	−23	Autopsy; polyneuritis.
250	184	26	−42	Spastic. Autopsy; polyneuritis.
	185	23	−60	
	186	21	−18	Crop full. Autopsy, beginning polyneuritis.
500	187	42	+8	Crop full; died.
	188	49	+40	Autopsy; polyneuritis.
	189	56	−25	" "

TABLE III.

Osborne and Wakeman's Fraction II from Bakers' Yeast No. 3.

Amount.	Bird No.	Length of life.	Change in weight.	Remarks
mg.		*days*	*gm.*	
75	157	35		Died.
	158	49	−47	Spastic; died.
	159	70	−18	Autopsy; mild polyneuritis.
125	161	70	−39	Well.
	162	70	−30	"
175	163	70	0	"
	164	39	−30	Autopsy; beginning polyneuritis.
	165	70	−30	Well.

TABLE IV.

Bakers' Yeast No. 3 on Pigeons.

Amount.	Bird No.	Length of life.	Change in weight.	Remarks.
		days	*gm.*	
200 mg.	101	70	−8	Apparently healthy.
	102	70	−20	Autopsy; normal.
	103	53	−72	" polyneuritis.
500 mg.	104	70	−35	Well.
	105	70	−11	"
	106	70	+64	"
1 gm.	107	70	−8	"
	108	70	+42	"
	109	70	+4	"

TABLE V.

Bakers' Yeast No. 9 on Pigeons.

Amount.	Bird No.	Length of life.	Change in weight	Remarks.
		days	*gm.*	
200 mg.	54	21	−34	Spastic; died.
	55	40	0	Crop full; died.
	56	35	−10	Died suddenly.
	57	34	+9	Paralyzed; died.
500 mg.	80	28	−10	Atropic; died.
	81	28	−2	Spastic; died.
	82	25	+28	Crop full; died.
1 gm.	97	49	+5	Autopsy; beginning polyneuritis.
	98	77	+17	" normal.
	99	70	+15	Healthy.

TABLE VI.

Osborne and Wakeman's Fraction II from Bakers' Yeast No. 9.

Amount.	Bird No.	Length of life.	Change in weight.	Remarks.
mg.		*days*	*gm.*	
75	139	23	−10	Autopsy; polyneuritis.
	140	49	−94	Weak; returned to normal on 200 mg.
	141	25	−60	Autopsy; polyneuritis.
125	142	70	−80	" very beginning of polyneuritis.
	143	70	−12	
	144	33	−49	Autopsy; mild polyneuritis.
175	145	42	−31	Died.
	146	28	−85	Autopsy; polyneuritis.
	147	70	+6	Apparently healthy.

TABLE VII.

Brewers' Yeast on Pigeons.

Amount.	Bird No.	Length of life.	Change in weight	Remarks
		days	*gm*	
200 mg.	67	34	Crop full.	Convulsions; died.
	69	30	−25	" "
	70	20	+45	Crop full. Autopsy; polyneuritis.
500 mg.	86	63	+36	Well.
	87	63	−13	"
	88	63	−18	"
1 gm.	89	70	+1	"
	90	70	−8	"
	91	70	+46	"

TABLE VIII.

Osborne and Wakeman's Fraction II from Brewers' Yeast.

Amount.	Bird No	Length of life.	Change in weight.	Remarks.
mg.		*days*	*gm.*	
75	130	65	−29	Autopsy; normal.
	131	65	−2	" polyneuritis.
	132	35	−43	Died.
125	133	63	+8	Autopsy; normal.
	134	63	+25	" "
	135	63	+25	" "
175	136	65	+3	" "
	137	65	−8	" "
	138	65	−25	" "

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STROPHANTHIN.

II. The Oxidation of Strophanthidin.[1]

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Attempts to obtain information regarding the structure of strophanthidin have been limited to the experiments of Feist[2] and Windaus and Hermanns.[3] Although the former studied the effect of a number of oxidizing agents, the only definite result obtained by him was by the oxidation of strophanthidin or isostrophanthidin (strophanthidinic acid lactone) with permanganate in alkaline solution after saponification of the lactone group in each of these compounds. In each case, along with oxalic acid and other obscure, amorphous acids, the same crystalline dibasic acid (so called strophanthic acid) was isolated with the melting point 260.8°, to which Feist attributed the formula $C_{27}H_{38}O_9$. Windaus and Hermanns later confirmed the formation of this acid, but on the basis of analysis, titration, and the analysis of a dimethyl ester adopted the formula $C_{23}H_{30}O_8$, and suggested that its formation might be due to the oxidation of a terminal CH_3 group in the hydroxy acid, erroneously considered by them to be $C_{23}H_{32}O_6$. From the following, this view will be seen to be untenable.

We have also prepared this acid and, from its analysis and titration in the cold, we have confirmed the formula $C_{23}H_{30}O_8$. It is isomeric with the acid to be described later on. However, from its behavior toward alkali, we have found that this acid, contrary to Feist, still possesses a lactone group and is, therefore, a dibasic lactone acid. On acidification the lactone ring readily closes with the quantitative formation of the original dibasic acid. Further, the dimethyl ester does not react with ketone reagents, so that the conclusion seems justified that the carbonyl group of strophanthidin is oxidized in the process of the formation of this acid.

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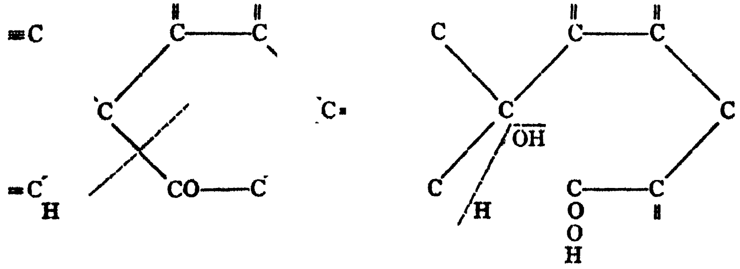
[1] Jacobs, W. A., and Heidelberger, M., *J. Biol. Chem.*, 1922, liv, 253.

[2] Feist, F., *Ber. chem. Ges.*, 1898, xxxi, 534; 1900, xxxiii, 2088.

[3] Windaus, A., and Hermanns, L., *Ber. chem. Ges.*, 1915, xlviii, 993.

The ester forms a benzoate. Accordingly, 7 of the 8 oxygen atoms contained in the acid have been accounted for. This would indicate that the main polycyclic skeleton of the strophanthidin molecule is composed only of carbon atoms. The relationship of this acid to strophanthidin is, at the moment, obscure, but the oxidation of the carbonyl group to carboxyl probably occurs in a manner similar to that discussed in the case of the following substance. Unlike the latter, the lactone ring is probably analogous to that contained in isostrophanthidin.

When strophanthidin is oxidized in the cold in acetone solution with permanganate, a monobasic acid is obtained, for which the formula $C_{23}H_{30}O_7 \cdot \frac{1}{2}H_2O$ has been derived. The analysis of the anhydrous substance was rendered difficult by the decomposition experienced when attempts were made to remove the water of crystallization. The above formula was confirmed by analysis of the silver salt and the methyl ester. From its behavior toward boiling alkali, the acid still possesses the lactone ring of strophanthidin. The methyl ester, however, no longer reacts with ketone reagents, and, in this respect, resembles the ester of the previously described dibasic acid. It is suggested, as pictured below, that the carbonyl group is attached to a tertiary carbon atom and, when oxidized, it is changed to carboxyl. The resulting tertiary alcoholic hydroxyl is then removed as water. At the same time 2 hydrogen atoms, elsewhere in the molecule, must be removed. Attempts to detect the presence of olefin linkings by reduction



of the ester with palladium and hydrogen were unsuccessful.

The ester forms a benzoate, so that the alcoholic group is presumably the same as that in strophanthidin which also forms a benzoate. Contrary, however, to the behavior of strophanthidin, which cannot be recovered after saponification of its lactone group, this acid may be, in part, recovered after such treatment.

Attempts to oxidize the above unsaponified acid in neutral solution with permanganate did not yield a tangible product. After saponification, however, this reagent produced a dibasic acid, $C_{23}H_{30}O_8$, isomeric with the previously discussed strophanthic acid of Feist, and Windaus and Hermanns. Contrary to the behavior of the latter, however, the new acid no longer contains the lactone ring. Instead, its dimethyl ester reacts with ketone reagents.

It may be concluded that the secondary alcoholic group contained in the lactone ring of the acid $C_{23}H_{30}O_7$, when once liberated by saponification to the dibasic acid, $C_{23}H_{32}O_8$, is oxidized to the carbonyl group of the acid $C_{23}H_{30}O_8$. Since strophanthidin possesses an analogous lactone ring, the internal ester of this compound is presumably likewise that of a secondary alcoholic hydroxyl group. The dimethyl ester of the acid $C_{23}H_{30}O_8$ still yields a monobenzoate.

Although, on boiling the above acid with alkali, no evidence was obtained of the presence of a lactone group, curiously enough when the alkaline mixture was reacidified, the original acid could not be recovered. Instead, a new dibasic acid was obtained with quite different properties. The analysis of the acid suggested the formula $C_{23}H_{32}O_9$. According to this, its formation was due to the addition of water to the molecule of the original acid. The new acid yielded a dimethyl ester which, in turn, gave an oxime and a monobenzoate. However, a constant error of about 0.6 to 0.7 per cent high in the carbon determinations on the acid, ester, and the benzoate will require explanation.

It is obvious that the oxidative degradation of strophanthidin presents the same difficulties which have been experienced with other members of the group of saturated polycyclic carbon compounds.

EXPERIMENTAL.

Oxidation of Strophanthidin.

The Acid $C_{23}H_{30}O_7$.—50 gm. of strophanthidin were dissolved in 2,000 cc. of acetone which had been carefully dried over calcium chloride and then distilled. The solution was turbined, chilled to 0–5°, and then treated with 25 gm. of powdered potassium permanganate. Oxidation occurred slowly with the gradual deposition of MnO_2 mixed with salts of the acid oxidation products. After about 2 hours, during which the temperature was maintained, the permanganate was completely used up. The collected precipitate was washed with acetone and then shaken up with water. The filtrate from MnO_2 was concentrated to about 150 cc. and acidified with acetic acid. The crude acid, which partly precipitated as a gum, slowly crystallized on standing and rubbing. The collected substance was suspended in water and dissolved by the addition of ammonia in slight excess. A very small amount of material, which proved to be unchanged strophanthidin, remained undissolved. The gently warmed solution on acidification yielded the acid as colorless rhombic leaflets. The yield was 13 gm.

The acetone filtrate from the above reaction mixture was concentrated to dryness. The resinous residue crystallized when treated with dilute ammonia. The crystals consisted of unchanged strophanthidin (6.7 gm.). The filtrate from the crystals, when concentrated and acidified with acetic acid, yielded a small amount of a gummy precipitate which was followed after seeding by a small quantity of the above described acid. No other crystalline product was obtained from the reaction mixture.

For analysis, the main fraction was recrystallized by dissolving in a small volume of hot 95 per cent alcohol and adding an equal volume of water. The acid slowly deposited as a crust of small, glistening, four-sided, stout plates or leaflets which contained, when air-dry, approximately 0.5 molecule of water of crystallization.

The substance softens above 175° and melts and effervesces at 185–190° although the melting point is considerably influenced by the rate of heating.

$$[\alpha]_D^{28} = +54.8° \ (c = 1.005 \text{ in methyl alcohol}).$$

It is readily soluble in methyl and ethyl alcohols, acetone, and acetic acid, and very sparingly soluble in chloroform, benzene, and ether. It dissolves completely in carbonate solution. In concentrated sulfuric acid it gives a yellow color which changes rapidly through orange and red to a permanganate purple.

The analysis was made somewhat uncertain by the difficulty of the complete removal of the water from the substance without accompanying decomposition.

Air-dry substance.

$C_{23}H_{30}O_7 \cdot \frac{1}{2}H_2O$. Calculated. C 64.60, H 7.31.
Found, (a). " 64.52, " 7.63.
(b). " 64.27, " 7.53.

Dried at 100° in vacuo over $CaCl_2$.

$C_{23}H_{30}O_7 \cdot \frac{1}{2}H_2O$. Calculated. H_2O 2.10.
Found. " 1.77.

Anhydrous substance.

$C_{23}H_{30}O_7$. Calculated. C 65.99, H 7.23.
Found. " 65.62, " 7.46.

The acid still possesses the lactone group contained in strophanthidin which was readily determined as follows:

0.1993 gm. of air-dry substance was dissolved in a few cc. of acetone, diluted with water, and titrated with 0.1 N NaOH, using phenolphthalein as an indicator. 4.50 cc. of alkali were required. The calculated volume for $C_{23}H_{30}O_7 \cdot \frac{1}{2}H_2O$ is 4.67 cc. A solution of 0.2007 gm. of the acid in 25.35 cc. of 0.1 N NaOH was refluxed for 45 minutes and then titrated back. 9.45 cc. of 0.1 N NaOH were used; 9.40 cc. are required by theory.

Strophanthidin, when once saponified by alkali, cannot be recovered as such, but only as the isomeric isostrophanthidin. The above acid, on the contrary, may be partly recovered after saponification. 0.5 gm. was dissolved in 10 cc. of 10 per cent NaOH and allowed to stand 24 hours at room temperature. On acidification with acetic acid, 0.2 gm. of the original acid was recovered as shown by melting point and properties. The mother liquor yielded a small additional amount after concentration.

Silver Salt.—As additional evidence for the formula adopted for the acid, the silver salt was prepared. The acid was dissolved in about 100 parts of water with a slight excess of ammonia which was then boiled off. The still hot solution when treated with silver nitrate solution yielded glistening, six-sided leaflets. The collected salt was suspended in water and dissolved by the careful addition of ammonia. The solution was then diluted and treated with acetic acid in slight excess. On standing, a small deposit of impurities formed from which the clear solution was filtered. After a few days in the refrigerator this deposited a crust of small plates and prisms which were stable to light.

Air-dry substance (dried at 100° in vacuo over H_2SO_4).

$C_{23}H_{29}O_7Ag \cdot 2H_2O$. Calculated. H_2O 6.42.
Found. " 6.65.

Anhydrous substance.

$C_{23}H_{29}O_7Ag$. Calculated. C 52.56, H 5.57, Ag 20.55.
Found, (a). " 52.41, " 5.72, " 20.45.
(b). " 20.67.

The Methyl Ester.—The above acid was esterified in acetone solution by diazomethane. The ester readily crystallized after removing the solvent. Recrystallized from methyl alcohol, it formed colorless, six-sided tablets which soften above 150° and slowly froth up at about 160–163°. It dissolves in alcohol, chloroform, and hot benzene, and less readily in ether. In concentrated sulfuric acid, the initial yellow color gradually deepens to an orange and finally to an orange-red with a purple fluorescence.

$$[\alpha]_D^{27} = 57.6° \ (c = 1.007 \text{ in methyl alcohol}).$$

Air-dry substance (dried in vacuo at 100° over H_2SO_4).

$C_{24}H_{32}O_7 \cdot H_2O$. Calculated. H_2O 4.00.
Found, (a) " 4.36.
(b). " 4.27.

Anhydrous substance.

$C_{24}H_{32}O_7$. Calculated. C 66.63, H 7.46.
Found, (a). " 66.66, " 8.10.
(b). " 66.49, " 7.63.

Attempts to prepare from the ester an oxime and a phenylhydrazone resulted only in the recovery of unchanged starting material. It may be concluded, therefore, that the carbonyl group contained in strophanthidin is no longer present in the acid obtained from it and the free carboxyl group in the latter is presumably formed by oxidation of this carbonyl group.

On the contrary, the formation of a benzoyl compound from the ester would suggest the retention of the alcoholic group of strophanthidin which yields a monobenzoate.

The Benzoate.—1.4 gm. of the methyl ester were dissolved in 20 cc. of dry pyridine and the solution after chilling was treated with 2 cc. of benzoyl chloride. After 1 hour, the mixture was poured into dilute sulfuric acid. The precipitated oil crystallized readily when treated with alcohol. After several recrystallizations from methyl alcohol, it formed colorless, minute prisms which melted at 243–244° (corrected). The substance dissolves easily in acetone and chloroform but is practically insoluble in benzene or ether.

$$[\alpha]_D^{20} = 61.0° \ (c = 1.004 \text{ in acetone})$$

$C_{31}H_{36}O_8$.	Calculated.	C 69.37, H 6.77.
	Found, (a).	" 69.10, " 6.76
	(b).	" 69.12, " 6.81.

Oxidation of the Acid $C_{28}H_{30}O_7$.

The Acid $C_{28}H_{30}O_8$.—Attempts to oxidize further the acid $C_{28}H_{30}O_7$ under conditions in which the lactone group had been left intact have thus far given unpromising results. After saponification, however, the liberated secondary alcoholic group was readily oxidized by permanganate to a carbonyl group.

10 gm. of the acid $C_{28}H_{30}O_7 \cdot \frac{1}{2}H_2O$ were dissolved in 100 cc. of 4 per cent NaOH. The solution was allowed to stand at 25° for 1 hour for saponification and was then diluted to 1,000 cc. 200 cc. of 5 per cent permanganate were added to the turbined mixture. The reaction rapidly completed itself, and the temperature rose to 27°. 100 cc. of normal acetic acid were then added and the mixture was filtered. The filtrate was concentrated to about 100 cc. under reduced pressure and then acidified strongly to Congo red with sulfuric acid. A partly crystalline precipitate slowly formed. After standing in the refrigerator for a day the collected acid was dissolved in a necessarily large volume of hot alcohol. Since the substance separated but incompletely on cooling, the filtrate was concentrated to about 100 cc. About 5 gm. of colorless, minute, flat needles or platelets separated. This material even after repeated recrystallization from alcohol gave analytical results which were 0.8 per cent high in carbon. This was unquestionably caused by a sparingly soluble by-product of the reaction. The acid was eventually purified over the barium salt which will be described below.

The barium salt, suspended in a small volume of water, dissolved when acidified with hydrochloric acid. On rubbing, the acid separated. The collected material was dissolved in dilute ammonia and again made to crystallize, by addition of acid. It formed, when allowed to crystallize undisturbed, minute, glistening, pyramided prisms which contained 2 molecules of water of crystallization. If allowed to crystallize rapidly, the acid may separate as globular aggregates of minute needles which are practically anhydrous. It crystallizes from alcohol in anhydrous form which melts and effervesces at 276–278° with preliminary sintering. The hydrate effervesces at 268–270° with preliminary sintering and discoloration. The latter dissolves fairly readily in hot water, but the anhydrous acid gradually separates. Otherwise, either form of the acid is difficultly soluble in the usual solvents. It dissolves in sulfuric acid with a yellow color which changes through orange to red with a green fluorescence.

$$[\alpha]_D^{28} = 28.0° \ (c = 0.995 \text{ in pyridine}).$$

Air-dry substance (dried in vacuo over H_2SO_4).

$C_{23}H_{30}O_8 \cdot 2H_2O$. Calculated. H_2O 7.66.
Found. " 8.17.

Anhydrous substance.

$C_{23}H_{30}O_8$. Calculated. C 63.56, H 6.96.
Found, (a). " 63.69, " 6.94.
(b). " 63.71, " 6.94.

0.1644 gm. of anhydrous substance was suspended in a small volume of water and titrated with 0.1 N NaOH, using phenolphthalein as indicator. 7.9 cc. were required. Calculated for the molecular weight 434.24, 7.6 cc.

0.0571 gm. of anhydrous substance was refluxed for 1 hour in 15.0 cc. of 0.1 N NaOH. 3.00 cc. were required. Calculated for two equivalents, 2.65 cc.

Although the titration after boiling with alkali seems to exclude the presence of a lactone group, the acid cannot be recovered as such but is converted into the acid below.

Barium Salt.—1.5 gm. of the above acid were dissolved in 150 cc. of water containing a slight excess of ammonia and the solution was treated with barium chloride solution. On concentrating under reduced pressure, the salt separated incompletely as glistening, silky needles.

The mother liquor yielded additional amounts. The salt was recrystallized by dissolving in hot water and since it showed little tendency to separate, the solution was concentrated to crystallization. The air-dried salt contained 6 molecules of water of crystallization. For the combustion it was necessary to use lead chromate.

Air-dried substance (dried in vacuo at 100° over H_2SO_4).

$C_{23}H_{28}O_8Ba \cdot 6H_2O$. Calculated. H_2O 15.95.
Found. " 16.30.

Anhydrous substance.

$C_{23}H_{28}O_8$ Ba. Calculated. C 48.46, H 4.92, Ba 24.12.
Found. " 48.26, " 5.16, " 24.77.

The Dimethyl Ester.—A suspension of the acid in acetone reacted at once with diazomethane. Evaporation of the solvent left a residue of rhombic platelets which were collected with ether. Recrystallized from methyl alcohol, the ester formed four-sided platelets and prisms which sintered at about 246° and melted with effervescence at 251–252°. The ester is soluble in alcohol, chloroform, and acetone, and very sparingly soluble in benzene or ether. It dissolves in concentrated sulfuric acid with a play of color through orange to deep red with a greenish fluorescence.

$[\alpha]_D^{22} = -12.0°$ ($c = 1.000$ in acetone).

$C_{25}H_{34}O_8$. Calculated. C 64.90, H 7.41.
Found, (a). " 64 84, " 7.27.
(b). " 64.79, " 7.15.

The Benzoate of the Dimethyl Ester.—This was prepared in the usual way from 1.5 gm. of the ester in pyridine solution with an excess of benzoyl chloride. After 1 hour, the reaction mixture when poured into acid gave a slowly crystallizing oil. The collected material was recrystallized from methyl alcohol, the operation requiring a large volume. 1.3 gm. of thin platelets, which melted with decomposition at 249–251°, were obtained. The substance dissolves readily in chloroform and pyridine, appreciably in acetone, and sparingly in hot alcohol. It is practically insoluble in ether and ligroin. In concentrated sulfuric acid it gives the same reaction as the original ester.

$[\alpha]_D^{20} = 7\ 5°$ ($c = 1.064$ in acetone).

$C_{32}H_{38}O_9$. Calculated. C 67.81, H 6.76.
Found, (a). " 67.73, " 6.61.
(b). " 67.96, " 6.57.

The Phenylhydrazone of the Dimethyl Ester.—1.7 gm. of the ester were heated on the water bath with 20 cc. of acetic acid and 2 gm. of phenylhydrazone. Stout crystals separated after 30 minutes. The collected substance was washed with acetic acid. Recrystallized from methyl alcohol, it formed lustrous platelets with a slightly yellowish tinge which melted and decomposed at 265–266°. It is sparingly soluble in the cold in the usual solvents.

$C_{31}H_{40}O_7N_2$.	Calculated.	C 67.35, H 7.30.
	Found, (a).	" 67.58, " 7.27.
	(b).	" 67.75, " 7.27.

The Oxime of the Methyl Ester.—1 gm. of the ester was refluxed for 2 hours in 20 cc. of methyl alcohol together with 0.5 gm. of hydroxylamine hydrochloride and 2 gm. of sodium acetate. The alcohol was boiled off and the residue made to crystallize by the addition of water. From dilute methyl alcohol lustrous, flat needles were obtained which are appreciably soluble in alcohol, acetone, and chloroform, and but little soluble in ether or benzene. It melts with decomposition at 272–274° with preliminary darkening and sintering.

$C_{25}H_{35}O_8N$.	Calculated.	C 62.85, II 7.39.
	Found, (a).	" 63 00, " 7.48.
	(b).	" 62.97, " 7.40.

The Acid $C_{23}H_{32}O_9$.—9.5 gm. of the above described acid, $C_{23}H_{30}O_8$, were dissolved in 100 cc. of 2 per cent sodium hydroxide solution and the mixture was then heated on the water bath for 30 minutes. When acidified with 25 per cent sulfuric acid, the original substance was not obtained but a new acid separated on rubbing as a thick paste of delicate needles. The collected acid was washed with ice water. The yield was 7.6 gm. Recrystallized from a small volume of water, the substance separates as colorless, glistening needles, containing when air-dry, 2.5 molecules of water of crystallization. It melts and effervesces at 185–187°. It dissolves easily in alcohol and appreciably in water and acetone. In sulfuric acid it gives at first a yellow color which changes to a red with green fluorescence.

Air-dry substance.

$[\alpha]_D^{28} = -37.0°$ ($c = 1.206$ in pyridine).

Air-dry substance (dried at 100° in vacuo over H_2SO_4).

$C_{23}H_{32}O_9 \cdot 2\frac{1}{2}H_2O$. Calculated. H_2O 9.05.
Found. " 8.76.

Anhydrous substance.

$C_{23}H_{32}O_9$. Calculated. C 61.03, H 7.13.
Found, (a). " 61.63, " 7.02.
(b). " 61.75, " 7.15.

After recrystallization from water, the analytical figures remained unchanged. The high carbon values for this acid, as well as the similar high carbon values which were obtained on analysis of the ester to be described below and the benzoate obtained from it, are at present difficult to explain. The discrepancy is not great, but it has appeared too consistently to be ignored. The analysis of the oxime, however, has given figures in better agreement with the postulated formula. For the present, we see no reason against the provisional acceptance of the formula $C_{23}H_{32}O_9$ for the acid. According to this view, it is formed by the addition of a molecule of water to the acid $C_{23}H_{30}O_8$. That this is not due to the opening up of a lactone was shown above by the titration of the latter acid before and after boiling with excess alkali. In accordance with this, the acid $C_{23}H_{32}O_9$ when titrated gave figures for a dibasic acid. 0.0890 gm., when suspended in water, required 4.30 cc. of 0.1 N NaOH, using phenolphthalein as an indicator. The calculated volume is 3.95 cc. for a dibasic acid $C_{23}H_{32}O_9$.

Until further data are obtained, it will be difficult to interpret the relationship of this acid to that from which it was obtained.

The Dimethyl Ester.—A suspension of the acid in acetone reacted smoothly with diazomethane with the formation of a clear solution. After removing the acetone, the ester, which slowly crystallized, was collected with ether. The addition of water to the solution in methyl alcohol caused the gradual deposition of a crust of glistening prisms. Recrystallized again for analysis, the ester melted at 205–206°. Repeated recrystallization as above or from dry acetone did not alter the analytical result, the carbon, as in the case of the acid, being about 0.6 per cent too high. The substance is soluble in alcohol, chloroform, and acetone, and but sparingly so in benzene and ether. In sulfuric acid it gives the same play of colors shown by the acid. In acetone solution, it showed no appreciable rotation, c = 1.000.

$C_{25}H_{36}O_9$.	Calculated.	C 62.46,	H 7.52.
	Found, (a).	" 63.06,	" 7.72.
	(b).	" 63.06,	" 7.69.

The Benzoate of the Dimethyl Ester.—1.5 gm. of the ester were benzoylated in 20 cc. of pyridine with 3 cc. of benzoyl chloride. After standing 1 hour the mixture was poured into an excess of dilute sulfuric acid. The pasty precipitate, which could not be made to crystallize, was shaken out with ether. The ether extract, washed successively with acid, water, dilute carbonate solution, and finally water, was dried and concentrated. The colorless syrup slowly, but only partly, crystallized on standing, which was facilitated by the addition of ether. 0.6 gm. was obtained. Recrystallized by dissolving in a small volume of methyl alcohol and adding an equal volume of water, it formed microscopic plates and prisms which melted at 172–174°. The substance is readily soluble in alcohol, acetone, chloroform, and benzene, and with difficulty in ether.

$C_{32}H_{40}O_{10}$.	Calculated.	C 65.72,	H 6.90.
	Found, (a).	" 66.33,	" 6 94
	(b).	" 66.30,	" 6.99.

The Oxime of the Dimethyl Ester.—A mixture of 1.5 gm. of the ester, 0.5 gm. of hydroxylamine hydrochloride, and 2 gm. of sodium acetate in 20 cc. of methyl alcohol was refluxed for 2 hours. Concentration of the solution and addition of water yielded a gum which crystallized after a day or so. Recrystallized by addition of water to its methyl alcoholic solution, and seeding, it forms minute, rhombic prisms which when air-dry contain 1 molecule of water of crystallization. This is held very tenaciously and it required rather long heating under reduced pressure over sulfuric acid to remove the water completely for analysis. The substance melts and slowly froths up at 158–160° with preliminary sintering. It is soluble in alcohol and acetone, and very sparingly in chloroform, benzene, and ether.

Air-dry substance (dried at 100° in vacuo over H_2SO_4).

$C_{25}H_{37}O_9N \cdot H_2O$.	Calculated.	H_2O 3.51.
	Found.	" 3.30.

Anhydrous substance.

$C_{25}H_{37}O_9N$. Calculated. C 60.57, H 7.53.
Found, (a). " 60.38, " 7.69.
(b). " 60.36, " 7.49.

Oxidation of Strophanthidin after Saponification.

Strophanthic Acid.—In all attempts to oxidize strophanthidin by the method of Feist, *i.e.* permanganate in alkaline solution, we have obtained an obviously impure amorphous acid as the main product of the reaction. The crystalline strophanthic acid of Feist was isolated only in relatively small amount. Since strophanthidin is easily subjected to a rather obscure alteration on boiling with alkali we have attempted to avoid this as much as possible by opening up the lactone group by the method used by Windaus and Hermanns in their titration of this substance. After many experiments the following method was found to give the best results.

50 gm. of strophanthidin were refluxed for 1 hour in a mixture of 5,000 cc. of alcohol and 3,750 cc. of 0.1 N sodium hydroxide solution. The solution was then concentrated under diminished pressure to remove all alcohol, diluted to 5 liters, and then oxidized by the gradual addition of 900 cc. of 5 per cent permanganate solution to the turbined mixture. Toward the end, the permanganate disappeared very slowly. The filtrate was acidified with acetic acid and concentrated under diminished pressure to about 200 cc. The further addition of acetic acid in excess was followed after seeding by the slow deposition of the crystalline acid which was accelerated by warming the mixture. The collected acid was washed with a little 50 per cent acetic acid and then with water. The yield was 7 gm. Acidification of the mother liquor to Congo red with sulfuric acid caused a gummy precipitate to form which could not be made to crystallize. The crystalline acid was suspended in a small volume of 50 per cent alcohol, dissolved by the addition of ammonia, and then reprecipitated with dilute sulfuric acid. Recrystallized again from dilute alcohol, it formed needles which melt and effervesce at about 270° (260.8° according to Feist). To the properties recorded by Feist we may add that in concentrated sulfuric acid it gives a yellow color changing gradually through orange to red. In methyl alcohol $[\alpha]_D^{20}$ is $- 22.0°$ ($c = 1.000$).

$C_{23}H_{30}O_8$. Calculated. C 63.56, H 6.96.
Found, (a). " 63.43, " 7.33.
(b). " 63.45, " 7.30.

From the analytical data we have adopted, in agreement with Windaus and Hermanns, the formula $C_{23}H_{30}O_8$ for the acid. Contrary to Feist, however, the behavior of the substance towards alkali has shown it to be a lactone acid. The explanation postulated by Windaus and Hermanns, that the substance originates by the conversion of a CH_3 group to COOH in the acid obtained by saponification of strophanthidin, is therefore untenable.

0.2000 gm. was suspended in·water and dissolved in the cold by the addition of a slight excess of 0.1 N NaOH. On titrating back to phenolphthalein, 9.10 cc. were used. The calculated amount for a dibasic $C_{23}H_{30}O_8$ acid is 9.18 cc. The opening of the lactone group requires somewhat more vigorous treatment than in the method used by Windaus and Hermanns for strophanthidin. 0.2004 gm. of the acid refluxed for 2 hours in 25.0 cc. of 0 1 N NaOH consumed 14.00 cc. of the alkali. The calculated amount for 3 COOH is 13.80 cc. On acidification of the mixture, the original acid rapidly crystallized and was almost quantitatively recovered. This is contrary to the behavior of strophanthidin which is converted, under these conditions, into an isomeric modification, isostrophanthidin. We believe that this acid is in reality an oxidation product of the latter. Since the ester no longer reacts with ketone reagents, the carbonyl group is no longer present. However, the formation of a benzoate shows the retention of an alcoholic group. In this acid four of the eight oxygens present are accounted for in the two carboxyls, two in the lactone group, and one in an alcoholic group.

The Dimethyl Ester.—This was prepared with diazomethane in accordance with Windaus and Hermanns. These workers report a melting point of 214°. Our substance melted at 251–253°. The substance was soluble in alcohol, acetone, and chloroform, and with difficulty in benzene and ether. In experiments to prepare the phenylhydrazone the ester was recovered unchanged.

$[\alpha]_D^{20} = -28.0°$ ($c = 0.995$ in methyl alcohol).

$C_{25}H_{34}O_8$. Calculated. C 64.90, H 7.41.
Found, (a). " 64.90, " 7.63.
(b). " 64.70, " 7.51.

The Benzoate of the Dimethyl Ester.—This was prepared as usual from the ester in pyridine with benzoyl chloride. Recrystallized from methyl alcohol, it formed glistening prisms which melt at 233–235°. It is soluble in chloroform and acetone, and particularly on warming in alcohol, ether, or benzene. The solution in acetone quickly deposits delicate needles which probably contain solvent.

$[\alpha]_D^{20} = -7.0°$ (c = 1.007 in acetone).

$C_{32}H_{38}O_9$.	Calculated.	C 67.81, H 6.76.
	Found, (a).	" 67.70, " 6.69.
	(b).	" 67.70, " 6.83.

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STROPHANTHIN.

III. Crystalline Kombe Strophanthin—Preliminary Note.

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Feist,[1] in 1900, was the first to attempt a careful study of the carbohydrate contained in strophanthin. His material, which was obtained from C. F. Boehringer Sons of Waldhof, was supposedly Kombe strophanthin. The conclusion was reached that the sugar contained in the drug is the methyl ester of a disaccharide of mannose and rhamnose. More recently, in 1913, Brauns and Closson[2] worked with identified Kombe seeds and were unable to confirm the findings of Feist. On distillation with acid, crystalline Kombe strophanthin as well as several commercial samples failed to give any appreciable amounts of methyl furfural. The conclusion was reached that Kombe strophanthin contains no rhamnose. However, no suggestion was offered as to the real nature of the sugar

In the year previous to the appearance of the above work, Heffter and Sachs[3] published their study of strophanthin prepared both from identified *Strophanthus hispidus* and commercial Kombe seeds. They were unable to prepare an osazone from the strongly reducing sugar fraction obtained from either of these glucosides. For this no explanation was offered.

Following certain preliminary observations we obtained a quantity of seeds from the open market which gave uniformly the green color with sulfuric acid which is regarded as characteristic for the genuine Kombe seed. From this material the crystalline glucoside, which agreed in all properties with those described by the above workers, was readily isolated.

In addition, however, it was found that the substance, when dissolved in acetic acid containing ferrous sulfate and then treated with a few drops of sulfuric acid, quickly yielded a deep purple solution which, on slight dilution with water, changed to a blue.[4] The same test was given by a sample of crystalline Kombe strophanthin kindly furnished us by Parke, Davis and Company from the material which had been prepared by Brauns and Closson from identified Kombe seeds. The positive outcome of the Kiliani reaction at once placed the sugar of Kombe strophanthin, in all probability, in the group of the desoxy compounds, digitoxose and cymarose. This is also supported by the formation of greenish flocks when the sugar solution was boiled with strong hydrochloric acid.

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[1] Feist, F., *Ber. Chem. Ges.*, 1898, xxxi, 534; 1900, xxxiii, 2069.

[2] Brauns, D. H., *J. Am. Pharmaceut. Assn.*, 1913, ii, 489, 604. Brauns, D. H., and Closson, O. E., *J. Am. Pharmaceut. Assn.*, 1913, ii, 715.

[3] Heffter, A., and Sachs, F., *Biochem. Z.*, 1912, xl, 83.

Up to the present, our attempts to prepare the sugar in crystalline form have been unsuccessful. The failure to obtain an osazone is in agreement with the view that the sugar is a desoxy compound. The methyl group in strophanthin is most likely contained in the sugar in the form of methoxyl, as in the case of cymarose, the sugar which Windaus and Hermanns[5] prepared from cymarin.

Attempts to prepare a crystalline phenylhydrazone or substituted phenylhydrazone have been thus far unsuccessful.

From the formula which we have adopted for strophanthidin, $C_{23}H_{32}O_6$, and on the basis of analytical data obtained with strophanthin itself, it is suggested that crystalline Kombe strophanthin possesses either the formula $C_{32}H_{48}O_{12}$ or $C_{31}H_{46}O_{11}$. In accordance with this the sugar is either $C_9H_{18}O_7$ or $C_8H_{16}O_6$ or the methyl ether of a C_8 or a C_7 desoxy sugar, respectively. An attempt is being made to obtain crystalline derivatives of the sugar.

EXPERIMENTAL.

Commercial strophanthus Kombe seeds which gave uniformly the green color test with sulfuric acid were worked up essentially according to the method used by Brauns and Closson with the exception that, after clearing the solution with basic lead acetate and after subsequent removal of excess lead, the crystalline glucoside was crystallized directly by the cautious addition of ammonium sulfate in an amount just insufficient to salt out the amorphous, gummy strophanthin. After several days the crystalline, flocculent glucoside was filtered on a large funnel and washed with small portions of water. It was recrystallized at first by solution in alcohol, by addition of an equal volume of water, and then by concentration under diminished pressure. When the alcohol was sufficiently removed, the glucoside crystallized readily as a mass of long, delicate, curved micro needles which were difficult to filter and wash. After several recrystallizations the glucoside was recrystallized directly from water, a process which, as stated by Brauns and Closson, is accompanied by considerable loss. The glucoside then formed lustrous, long, thin, pointed platelets or when rapidly cooled, long, curved, delicate threads which were often radially grouped.

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[4] Kiliani, H., *Arch. Pharm.*, 1896, ccxxxiv, 273; 1913, ccli, 575.

[5] Windaus, A., and Hermanns, L., *Ber. chem. Ges.*, 1915, xlviii, 979.

The substance possessed the recorded properties and melted when rapidly heated at 180–183°, followed by a slow frothing. When a small amount was dissolved in acetic acid and then treated with a few crystals of ferrous sulfate, followed by a few drops of sulfuric acid, the solution gradually developed a deep red-purple color. On addition of a small amount of water, this changed to a blue and finally to green. This color resembles the reaction described by Kiliani for digitoxose and by Windaus and Hermanns for cymarose with the exception that they describe only a blue color. The identical reaction was given by a sample of Kombe strophanthin kindly furnished us by Parke, Davis and Company which had been prepared by Brauns and Closson from identified seed.

$[\alpha]_D^{29} = 30.5°$ (c = 1.02 in 95 per cent alcohol)

Air-dry substance (dried at 100° in vacuo over H_2SO_4*).*

$C_{32}H_{48}O_{12} \cdot 3H_2O$. Calculated. H_2O 7.96.
$C_{31}H_{46}O_{11} \cdot 3H_2O$. " " 8.36.
Found. " 7.60.

Anhydrous substance.

$C_{32}H_{48}O_{12}$. Calculated. C 61.50, H 7.75, OCH_3 4.97.
$C_{31}H_{46}O_{11}$. " " 62.59, " 7.80, " 5.22.
Found, (a). " 62 02, " 7.52.
(b). " 62.30, " 7.57.
(c). OCH_3 5.26.

0.4051 gm. of anhydrous substance was refluxed for 45 minutes in 25 cc. of 0.1 N NaOH and 25 cc. of water and then titrated against phenolphthalein. 6.7 cc. of alkali were used. Molecular weight is 605. Calculated for $C_{32}H_{48}O_{12}$ it is 624. For $C_{31}H_{46}O_{11}$ it is 594.

5 gm. of crystalline strophanthin were dissolved in a mixture of 40 cc. of 50 per cent alcohol and 10 cc. of concentrated hydrochloric acid and allowed to stand 4 to 6 hours in the cold. The mixture was then diluted and allowed to stand with occasional rubbing. 2.7 gm. of strophanthidin were obtained. The mother liquor was neutralized with pure barium carbonate and the filtrate concentrated under reduced pressure to dryness. The residue was extracted with alcohol and the filtrate from barium chloride was concentrated to dryness. On dissolving the syrup in water an additional 0.3 gm. of strophanthidin was recovered, the total yield amounting to 3 gm. The theory for C_{31} or C_{32} is roughly 3.2 to 3.3 gm.

In the sugar solution, the residual Cl ions were removed with silver sulfate and the filtrate was then treated with hydrogen sulfide. Sulfuric acid, remaining in the filtrate, was removed quantitatively with barium carbonate. The filtrate yielded on concentration a dextro-rotatory syrup which still possessed a somewhat bitter taste. This syrup could not be made to crystallize and attempts to prepare an osazone were fruitless. Likewise, a crystalline phenylhydrazone, bromophenylhydrazone, and benzylphenylhydrazone could not be obtained. The reducing substance was partly soluble in ether and benzene and, from its tendency to become resinous on standing, indicated its instability at least in the impure state.

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A WATER-JACKETED HYDROGEN ELECTRODE.

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(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received May 21, 1923.)

A hydrogen electrode cell has been designed to give rapid and accurate results and at the same time offer greater convenience in manipulation when working with certain fluids, than other types of cells. The results have been sufficiently satisfactory to warrant publication of a description of the cell.

DESCRIPTION OF CELL.

This cell is kept at constant temperature by means of a water jacket through which water from a thermostatic bath is circulated. It has a 3-bore, 4-way stopcock which performs all the functions required. This stopcock is bored as shown in Fig. 1. The three bores do not intersect. Bore *c* is used in both Position III and Position IV. In order that both ends of Bore *c* may coincide with the opening of the arm for the salt bridge, this opening of the arm must be elliptical in shape. The cell may be constructed of either Pyrex or ordinary glass.

The Platinum Electrode is a spiral of platinized platinum wire sealed into a glass tube passing through a rubber stopper to the bottom of the cell. The tube contains mercury in order to make contact. The stopper has a supplementary hole for the escape of hydrogen. Readings are taken with the electrode entirely immersed. The spiral serves, furthermore, to break up the bubbles of hydrogen, thus promoting rapid saturation. The electrodes are frequently changed and re-platinized. A fresh electrode must be saturated for a much longer period before the first reading is made. When not in use, the cell is kept filled with distilled water.

The Calomel Cell is constructed with a water jacket and has a platinum wire sealed into the bottom and a mercury contact. In it are placed mercury, calomel and mercury, and a solution saturated with both potassium chloride and calomel. It is filled to the top and when the stopper is introduced the excess solution is forced out of the tube (which contains a loose plug of cotton), thus eliminating all air bubbles.

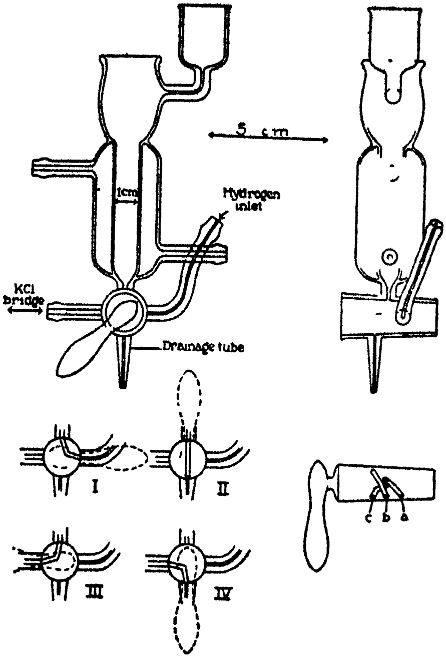

Fig. 1.—The water-jacketed hydrogen electrode cell. In Position I, Bore *a* connects the source of hydrogen with the cell; in Position II, Bore *b* connects the cell with the drainage tube; in Position III, Bore *c* connects the cell with the salt bridge; in Position IV, Bore *c* connects the salt bridge with the drainage tube. Order of manipulation: I, II, IV, III.

The Salt Bridge is "closed," that is, not open to the atmosphere's pressure. When the liquid junction is formed there can be no flow of liquid. Saturated potassium chloride solution is used to eliminate liquid junction potential and, for convenience, is colored with α-azurine G. A reservoir (D) of this solution is kept at constant level by means of a glass tube connected with an inverted bottle of potassium chloride solution on a shelf above the apparatus, and is connected to the bridge by means of a stopcock (E) which is kept closed by an elastic band attached to a glass hook fused to the stopcock handle. One liter of potassium chloride solution will serve as a supply for a year.

The Hydrogen, which is supplied from a tank with a reducing valve, is passed through water and then through a coil in the constant-temperature bath, from which it is brought to the electrode by means of Tube J. The pressure of the hydrogen is regulated to about 6-7 mm. of mercury. This gives an even flow of small bubbles when the stopcock (in Position I) is partly opened.

Fig 2.—Hydrogen-ion apparatus. A Potentiometer. B Galvanometer. C. Storage battery, single cell D Constant level reservoir of saturated potassium chloride solution E Self-closing stopcock connecting the potassium chloride reservoir with the salt bridge. F. Water-jacketed calomel cell. G. Salt bridge. H. Hydrogen electrode cell (of a different type than described in this article) I *Hydrogen electrode cell* described in this article. J. Hydrogen gas supply. K Dish to collect drainage L. Automatic nipple for distilled water supply.

Water Circulation from the bath through the cells is accomplished by means of an "air lift" which is merely a siphon system through the cells into which air is injected to raise the water back up to the bath.

It is not essential for the functioning of the air lift that the bath be at a higher level than the cells. If the bath were on the same level as (or even below) the cells, the water after passing through them could be carried down to the floor and then raised to the bath by means of the air lift, thus circulating the water.

MANIPULATION.

The hydrogen electrode cell has but a single stopcock which performs the following functions. *First*, in Position I, it allows hydrogen to be bubbled through the apparatus (for three minutes). *Second*, in Position II, it permits a few drops of solution to drain, and thus remove all bubbles of hydrogen.[1] *Third*, it is swung around (to the left) to Position IV, connecting the salt bridge with the drainage tube. Here no liquid can flow until Stopcock E is opened. Hence the stopcock of the cell is left open in Position IV while the operator reaches with the same hand to open Stopcock E, permitting a few drops of potassium chloride solution to drain. Thereafter it is necessary merely to release Stopcock E, since it closes automatically. This procedure has filled Bore *c* of the cell stopcock with potassium chloride solution. *Fourth*, it is then turned to Position III, thus forming the liquid junction, and a reading is taken. If another reading is desired, the stopcock is turned to Position II, where a few drops of solution are allowed to drain, and then turned to Position I, where the saturation with hydrogen is continued.

DISCUSSION.

This cell has the following advantages.

1. Its temperature may be accurately controlled without interfering with its manipulation. It may be more conveniently handled than is the case with cells placed in an air or oil thermostat.

2. The temperature of the cell is maintained with such constancy that a solution which is much colder or much warmer than the cell

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[1] In case the drainage of the solution (in Position II) is stopped by bubbles, the hole in the rubber stopper is closed with the forefinger of the left hand, while the fleshy portion of the right thumb is gently pressed on the filling cup of the cell. The pressure created will start the flow of liquid.

will be brought to the desired temperature during the three minutes required for saturation. Thus, a solution may be taken from the refrigerator and introduced directly into the cell without being previously warmed.

3. One stopcock performs all the functions required, thus preventing accidental passage of gas into the salt bridge or of potassium chloride solution into the cell.

4. The closed bridge prevents mechanical agitation when the liquid junction is formed.

5. The liquid junction is only 6 mm. from the platinum electrode, thus permitting accurate readings even with solutions of low conductivity.

6. One to two cc. of solution is sufficient for 2 to 6 consecutive readings.

Accuracy.—Bubbling with hydrogen for three minutes gives readings which do not change more than 0.1 to 0.2 millivolt even after two hours' bubbling. Readings are reproducible to 0.01 pH.

The fact that the whole system is not at the same temperature should produce no appreciable error, since the liquid junction is at practically the same temperature as the electrodes and no potential can be produced by temperature variations in the bridge.

The "open stopcock" junction is subject to the usual errors.

SUMMARY.

A simple water-jacketed hydrogen electrode cell of the bubbling type is described which is accurately maintained at constant temperature with water from a bath, circulated by means of an air lift.

It gives accurate readings reproducible to 0.01 pH with 1 or 2 cc. of solution after three minutes' saturation with hydrogen, even with solutions of low conductivity.

It has a single stopcock which performs separately the following functions: It permits (1) bubbling of hydrogen gas through the solution; (2) drainage of solution; (3) drainage of potassium chloride solution from the salt bridge; (4) formation of liquid junction.

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A METHOD FOR THE PHYSIOLOGICAL STUDY OF TISSUES IN VITRO.

BY ALEXIS CARREL, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 30 AND 31.

(Received for publication, May 21, 1923.)

The aim of the method described in the present paper is to maintain tissues in a condition of uninterrupted growth in a medium which does not deteriorate spontaneously. This simple idea has doubtless occurred to many experimenters, but great technical difficulties have so far prevented the development on this principle of a method of cultivating tissues. The problem consists of giving the cells the necessary food material and removing the catabolic substances from the medium without disturbing the tissues and without bacterial contamination. It has been solved by the construction of containers and instruments permitting the aseptic handling of the cultures, and by the use of a medium composed of two parts, solid and fluid, the solid medium being continually bathed by the fluid medium which is changed as often as necessary.

I.

Preparation and Handling of the Cultures.

1. Containers and Instruments.—The containers are flat, round flasks with narrow, oblique necks through which tissues and media may be introduced and removed. The neck is 3 cm. long and 1 cm. wide, and can be easily sterilized in a Bunsen flame. The width of the neck and its inclination are such that tissues and fluids are easily introduced with a pipette, and particles of dust cannot fall into the culture medium while the flask is opened. It is closed with absorbent cotton and a rubber cap. The flasks belong to five types (Fig. 1), and each type may be made in two sizes, 5 cm. or 8 cm. in diameter.

Type A bears a top opening 3 cm. in diameter, and is used for the cultivation of tissues which cannot be handled conveniently through the neck of the flask. The opening is closed by a disc of glass, fixed with shellac. Type B has two necks at the opposite sides of equal diameter and no top opening. It is convenient when the preparation of the culture requires the handling of the tissues with two instruments, which are introduced through the opposite necks. Type C has a long neck, a bottom opening closed by a thin mica plate, and also a top opening. It may easily be inverted under a microscope for photography or examination under high power of the living tissues. After the culture has been fixed in formaldehyde, the bottom of mica is excised with ordinary scissors or unsealed without disturbing the solid medium. Then the tissues and their medium, adherent to the thin mica plate, may be stained and studied under high magnification. Type D has only one neck and is commonly used for the cultivation of fibroblasts, epithelium, leucocytes, and virus, and for the study of cell secretions. Type E resembles Type D, and possesses a bottom opening covered with mica. Flasks A, B, and D are made of plain glass which allows the tracing of the cultures in a projectoscope and also low power microscopic examination. When cytological studies are required flasks of Types C and E are used.

The tissue fragments are handled and introduced into the flask with long platinum spatulas, straight or curved, and mounted on a glass rod (Fig. 2). Other kinds of spatulas are used for cutting the solid medium, when part of the culture has to be removed from the flask. The introduction of the fluids into the flask is made with short, graduated pipettes with rubber nipples (Fig. 3). The fluids are removed through a large hollow needle 4 cm. long, connected by rubber tubing with a vacuum apparatus (Fig. 3). The aspirating needle is also used when aeration of the culture is needed.

2. *Culture Medium.*—The culture medium is composed of two parts, solid and fluid. The solid medium consists of a coagulum of fibrin obtained from plasma or fibrinogen. When fibrinogen forms the basis of the clot, the fibrin is protected from digestion by a little serum or, when serum cannot be used, by a small amount of sodium linoleate or suspension of egg yolk. The volume of the solid medium in a flask 5 cm. in diameter should be 2 cc. If it is larger, the coagulum

becomes too thick and the tissues are isolated from the fluid medium by a layer of fibrin through which the diffusion of nutrient and catabolic substances is very slow. The volume of the fluid medium is generally 1 cc. The asepsis of the fluids used in the composition of the medium must be ascertained by the proper bacteriological tests, and the H ion concentration by the colorimetric method.

The plasma is introduced first. Generally 0.5 cc. of plasma is injected into the flask, which is gently tilted in order that the whole bottom may be moistened. This prevents the loosening of the coagulum from the glass surface, which may otherwise occur after a few days. Then 1.5 cc. of Tyrode solution, containing 5 per cent tissue extract, is introduced and mixed with the plasma. When a fibrinogen suspension is used instead of plasma, it is introduced in the same manner. To 0.5 cc. of fibrinogen suspension is added 0.5 cc. of doubly concentrated Tyrode solution. Then 0.5 cc. of Tyrode containing a little sodium linoleate or serum, and 0.5 cc. of dilute embryonic tissue extract, are introduced. Before coagulation takes place, the fragments of tissues, carried on a platinum spatula, are placed in the medium. If there are many fragments, they are suspended in Tyrode solution and injected with a pipette. After coagulation has taken place, the fluid medium is poured on the surface of the clot, and the neck of the flask is flamed and tightly closed.

The fluid medium must be changed every 2nd, 3rd, 4th, or 5th day, according to the nature of the medium and the tissues. The flasks are brought into a room where the air has been sprayed and is practically free of dust. The rubber caps are removed and the neck is carefully flamed. Then the fluid is withdrawn by means of the aspirator (Fig. 3) or a pipette, and the new fluid introduced. The neck is again flamed and closed. The time required for changing a complex medium varies from 45 to 75 seconds. It is generally possible to handle about 60 flasks in 1 hour. If a fragment of the culture has to be transferred to another flask, the coagulum is detached with a spatula and removed through the neck of the flask. Then the tissues are dissected and a new culture is made.

3. Measurement of the Rate of Growth and Examination of the Cells.—The flasks are placed in a projectoscope and the outline of the tissue is traced every 1 or 2 days. As the thickness of the solid medium and the density of the fibrin meshwork are about uniform, the increase in area of the tissue expresses the increase of its volume with some accuracy. When the area is plotted in ordinate and the time in abscissa, the curve expressing the growth of connective tissue or of epithelium is a parabola.

Microscopic examination of the tissue can be made with low power through the bottom of Flasks A, B, and D, and with high power through the mica sheets of Flasks C and E.

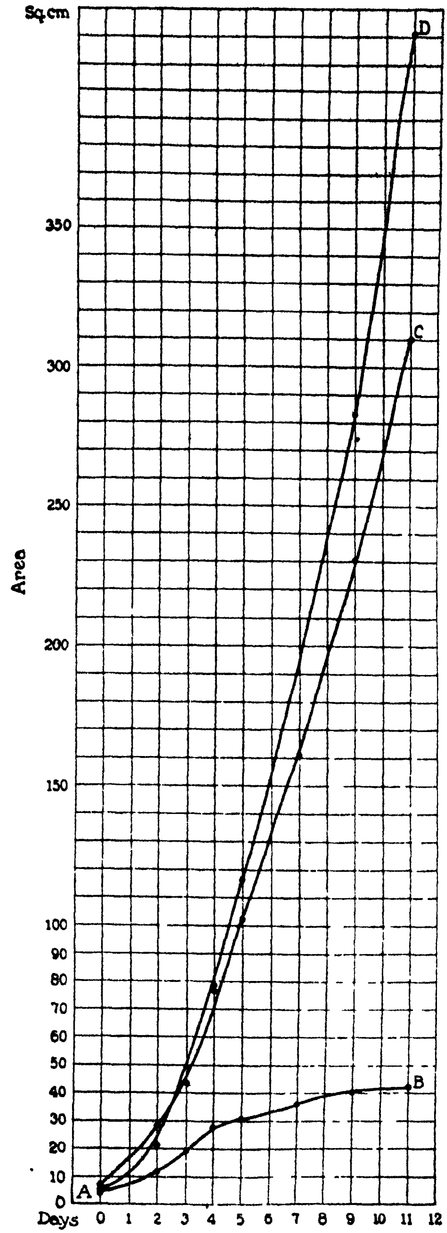
The tissues are fixed, the flask being still in the incubator, by warm Ringer solution containing 2 per cent formaldehyde. After fixation, the culture is washed and detached from the flask, then stained, or sectioned and stained. But the tissues are often damaged in the process of removal from the bottom of the flask. Therefore, when microscopic examination is needed, the tissues are grown on the bottom of Flasks C and E.

II.

Characteristics of the Growth.

The fibroblasts were obtained from an 11 year old strain of connective tissue, or from the heart of chick embryos. They were imbedded in the coagulum covering the bottom of the flask and bathed in the fluid medium. When the fluid medium was composed only of Tyrode solution or serum, the fibroblasts used the food material stored in the tissue fragment itself. Then, the rate of growth became slower and ultimately the tissues died. The duration of the life of the culture in this non-nutrient medium expressed the residual activity of the tissue and its graphic representation is generally a long S-shaped curve (Text-fig. 1, Curve *AB*). When the fluid medium contained some nutrient substances, the tissues increased regularly in mass (Fig. 4). The diameter of the new colony might reach 1.8 or 2 cm. after 2 or 3 weeks, and the curve of the growth was a parabola (Text-fig. 1, Curves *AC* and *AD*).

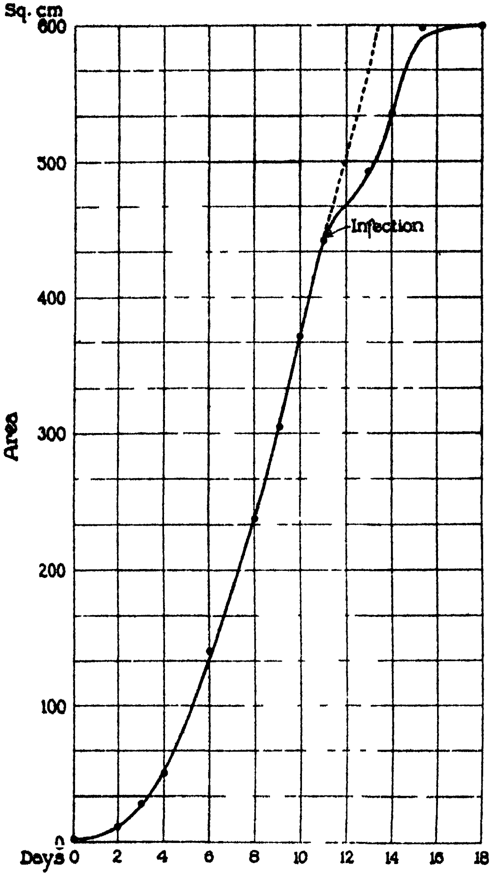
The rate of growth was modified by minute changes in the constitution of the medium. When bacterial contamination occurred, the rate of growth decreased and the tissues died (Text-fig. 2). Slight differences in the composition of the medium brought about marked differences in the rate of growth. During the first days of life of the



TEXT-FIG. 1. Curve *AB*, growth of fibroblasts in a medium containing no nitrogen. Curve *AC*, growth of fibroblasts in a medium containing 21 mg. of nitrogen per 100 cc. Curve *AD*, growth of fibroblasts in a medium containing 30 mg. of nitrogen per 100 cc.

cultures, these differences were not apparent, but after 2 or 3 weeks, they became evident (Text-fig. 1).

The changes in the rate of growth of a given tissue under the influence of another tissue could also be studied. For instance, when a colony of leucocytes, growing under the influence of serum, reached the edge of a resting culture of fibroblasts, these cells began to proliferate again.



TEXT-FIG. 2. Growth of a culture of fibroblasts which became infected on the 11th day and ultimately died.

Epithelial cells grew as a membrane at the surface of the coagulum. The curve of growth was also a parabola. The rate of growth of epithelium was always less rapid than that of fibroblasts.

Leucocytes from the blood and wandering cells from the spleen were cultivated in Flasks D and E. From spleen fragments, about 1 sq. mm. of wandering cells migrated and in 30 or 35 days covered an area of about 2,000 sq. mm.; that is, the entire medium. Leucocytes from small blood clots grew closely packed together and later formed a number of small colonies scattered through the medium. These colonies originated by the grafting on the surface of the coagulum of groups of cells which floated into the fluid medium. They never aggregated in a tissue, and an accurate measurement of the area invaded by them was not possible.

III.

Accidental Causes of Death.

When the solid medium remains unaltered, and the proper nutrient fluid is added to it from time to time, the tissues invade the coagulum progressively. But several factors may interfere with their growth and bring about premature death. The more important of these factors are bacterial contamination, changes in the H ion concentration of the medium, and alteration of the coagulum.

The first is generally due to the contamination of the fluids composing the medium during their preparation; to non-sterile instruments, pipettes, or spatulas; to contact between the fluid medium and the stopper of the flask, and finally to contamination from the air. Plasma is never contaminated because, being received directly into tubes, it is not exposed to air and contaminating contacts in the course of its preparation. As Tyrode solution is sterilized by filtration through a Berkefeld filter, it is almost never a source of contamination. Tissue juice is often responsible for contamination. However, the following technique usually yields an aseptic preparation. The embryos are extirpated carefully from the egg or from the uterus, and immediately introduced into a Latapie apparatus. Then, the pulp is received in a graduated flask and the necessary amount of Tyrode solution is added. After centrifugation, the extract is placed in a number of small tubes in order that the contents of each tube may be used entirely, after the tube is opened. The condition of each tube must always be tested by cultivation of a sample of fluid in bouillon and on agar. As some bacteria develop very slowly, these cultures have to be kept under observation for 5 or 6 days, before the fluids can be considered as sterile and used for the growth of tissues. Practically all the failures could be traced to a contamination of the tissue juice which had been overlooked in the examination of the bacterial cultures. The opening of the flasks, the aspiration of the fluid, and the introduction of new medium practically never give rise to contamination, because the neck of the flask and the spatulas are easily sterilized in a Bunsen burner flame, and the pipettes are easily kept aseptic. Contamination may also be traced to contact of the fluid medium with the cap when the flasks are handled without sufficient care. None occurs as long as the fluid medium does not penetrate the oblique neck during the transportation of the flasks. When the flasks are carried in a tray especially constructed, where they are placed vertically, with the neck up, there is no contact of the fluid with the stopper, and the number of bacterial contaminations is much lessened.

The infection by air appears to be very unusual. In the room where the flasks are opened and the fluid medium is replaced, the number of bacterial colonies found in half an hour on Petri dishes about 100 sq. cm. in area is only 3. As the section of the neck of the flasks is less than 1 sq. cm. and the time during which the flask is opened does not exceed 30 seconds, the chances of contamination by air are extremely small. Besides, if a few particles of dust should fall inside the neck, they would be destroyed by the flaming which always precedes the closing of the flask. When the air contained in the flasks is aspirated and replaced by the air from the room, contamination is also quite exceptional.

Another cause of death of the tissues is a change in the reaction of the medium. When the pH of the medium is higher than 8, the growth decreases and sometimes ceases entirely. The excess of alkali may come from the glass. Therefore, every new flask has to be tested from this point of view before being used. Generally the alkali comes from insufficient washing. A special apparatus has been made for rinsing the flasks after they have been cleansed with sodium hydroxide. Since its use, no losses due to excess of alkali have occurred. A more frequent source of accident is acid formation.

When the pH of the culture is lower than 7.2, the rate of growth is markedly modified and the tissues die. This is generally due to a spontaneous change of the tissue extract which occurs mainly when particles of tissues are suspended in the fluid. The H ion concentration of every fluid used in the cultures must be tested and adjusted to 7.6 or 7.8.

When the fluid medium is composed only of tissue juice, the fibrin may become partly digested after a few days. This accident is prevented by adding to the tissue juice a small amount of serum, which protects the fibrin against the proteolytic enzymes. Sodium linoleate or egg yolk may be substituted for serum. When the beginning of the digestion of the fibrin is observed, a few drops of plasma are placed at the surface of the coagulum and the tissues generally resume their normal growth.

IV.

DISCUSSION.

The elucidation of the fundamental relations between the tissues and the humors of the organism would require an analysis of the action of the serum constituents on pure strains of cells, of the effect of the secretions of those cells on the composition of serum, and of the influence of the tissues upon one another. The main requisites of a method permitting such an investigation are to maintain pure cultures of cells in a condition of uninterrupted growth in a medium of practically unchanging composition. We already possess techniques by which strains of fibroblasts,[1] epithelial cells,[2] and leucocytes[3] may be isolated and kept free from contamination by other cells. But these tissues must be maintained in a known condition; that is, in a medium which does not deteriorate spontaneously. As the activity of a tissue is a function of the concentration of certain substances in its medium,[4] it is evident that the effect of a given factor on cell proliferation or morphology cannot be ascertained if at the same time the medium undergoes spontaneous and ill defined modifications.

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[1] Ebeling, A. H., *J. Exp. Med.*, 1922, xxxv, 755.

[2] Fischer, A., *J. Exp. Med.*, 1922, xxxv, 367.

[3] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxvi, 365.

[4] Carrel, A., *J. Exp. Med.*, 1913, xviii, 287.

None of the techniques used so far fulfill these two main conditions. In the early method of so called cultivation of tissues, which is still employed by many experimenters, a fragment of fresh tissue, generally embryonic, is placed in a drop of plasma[5] or saline solution,[6] and for a few days migration and multiplication of cells may be observed. But the phenomenon is irregular and of short duration, and no increase in the mass of the tissues is observed. When the area of a pure culture of fibroblasts so cultivated is accurately measured, the curve of the growth resembles that of a unimolecular autocatalytic reaction. The second inflection of the S-shaped curve begins after 48 hours,[7] and is due to a change of the medium which greatly restrains the rate of growth and brings about the death of the cells. During 48 hours only, the growth is uninfluenced by the progressive deterioration of the medium. Later, the action of a given factor on the growth of fibroblasts cannot be studied on account of the simultaneous effect of the modifications of the medium on the tissues.

In order to prevent the effect of the deterioration of the medium, a method was developed long ago[8] in which the tissues were removed from the medium after 48 hours, washed in Ringer solution, and placed in a new medium. By the continual transfer of the tissues from medium to medium,[1] the cells were maintained in an identical condition, and the rate of growth of a strain of connective tissue, cultivated in this manner for more than 11 years, has not varied.[1] Today these fibroblasts multiply with the same velocity as 10 years ago. Many physiological problems have been investigated with the help of this technique. But it cannot be applied so effectively to more delicate structures such as epithelium and leucocytes. The amount of tissue is very small and the presence of secretions cannot be ascertained. Moreover, the section of the tissues and their transfer require a great deal of training and manual skill.[9] As the growth is interrupted every 48 hours, the prolonged action of a given factor must

[5] Carrel, A., and Burrows, M. T., *J. Exp. Med.*, 1911, xiii, 387.

[6] Lewis, M. R., *Anat. Rec.*, 1915–16, x, 287. Lewis, M. R., and Lewis, W. H., *J. Am. Med. Assn.*, 1911, lvi, 1795.

[7] Ebeling, A. H., unpublished experiments, 1919.

[8] Carrel, A., *J. Exp. Med.*, 1912, xv, 516.

[9] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.

be observed during a number of passages. Thus, it involves much labor, and the process of growth cannot be expressed graphically in a satisfactory manner. For these reasons, an attempt was made to develop a better method.

It seems that the technique described in this article, although far from perfect, may be applied to the solution of many problems and fulfill most of the conditions required for a physiological study of tissues *in vitro*. However, the medium deteriorates in some measure because the substances contained in the fibrin do not diffuse entirely into the fluid which covers its surface. The coagulum, incompletely washed by the fluid medium, may be slowly modified by the substances which progressively accumulate in its meshwork. But the parabola expressing the increase of the tissues indicates that the growth proceeds at a constant rate for 2 or 3 weeks, instead of 2 days as in the hanging drop technique. A culture of fibroblasts or of epithelium can be kept in a D flask for about 3 weeks in a condition of active life. This space of time is longer than necessary for the study of most physiological phenomena. Leucocytes can remain in excellent condition for at least 35 days,[10] and the coagulum is practically as transparent after this period of time as on the 1st day, although it may be completely invaded by the wandering cells. As fifteen or twenty fragments of tissues or of leucocytic film can be placed in a D flask containing 2 cc. of medium, the nature of cell secretions can be investigated in some measure.

The growth, being uninterrupted, may be represented graphically, and the part played by the constituents of the medium in the growth analyzed without difficulty. The characters of the curve vary according to the nature of the medium. In a nutrient medium the growth is expressed by a parabola and in a preservative medium by an S-shaped curve. This curve is the measure of the residual activity of a tissue. It is known that the activity of a tissue at a given instant is function of at least three independent variables, the inherent cell activity at the preceding instant, and the concentrations of growth-activating and growth-inhibiting substances in the medium. The inherent activity of a tissue may be defined as that which would be displayed during an instant in a medium deprived of all nutrient substances. The energy used by a tissue in that condition would be supplied by the food material stored in the cells or in the interstitial lymph, and not by the medium. This inherent activity cannot be measured directly. But it is certainly proportional to the residual activity; that is, to the increase in area of the tissue and to the duration of its life in a preservative non-nutrient medium. The residual activity being known, it is easy to measure the part which is played in the process of growth by the nutrient substances of the medium. The area *ABD* in Text-fig. 1 represents the increase of the tissue due to the action of the medium; that is, the amount of protoplasm built up by the cells from the nutrient substances contained in the medium.

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[10] Carrel, A., unpublished experiments, February, 1923.

There is no doubt that this method permits the measurement, conveniently and without great labor, of the effect of many factors on cell proliferation, and will therefore help in the study of physiological and pathological problems.

V.

CONCLUSIONS.

1. A method has been developed which allows the continuous growth of pure strains of fibroblasts, epithelium, and leucocytes in a medium which undergoes but slight spontaneous deterioration.

2. The principle of the method is to leave the tissues undisturbed while the medium is changed. This was realized by special containers allowing the change of the medium without bacterial contamination and by the simultaneous use of a solid and a fluid medium.

3. The curve of growth of pure cultures of fibroblasts and epithelial cells in a nutrient medium is a parabola; in a non-nutrient medium, it is S-shaped and expresses the residual activity of the tissues. Leucocytes invade the culture medium progressively, as do bacteria, but never aggregate in a tissue.

4. The method is used for the study of the morphological and dynamic changes occurring in tissues under the influence of chemical and physical factors.

EXPLANATION OF PLATES.

PLATE 30.

FIG. 1. Flasks A, B, C, D, and E.

FIG. 2. Introduction of tissues into a D-5 flask with a spatula.

PLATE 31.

FIG. 3. Aspiration of the fluid.

FIG. 4. Culture of fibroblasts in a D flask.

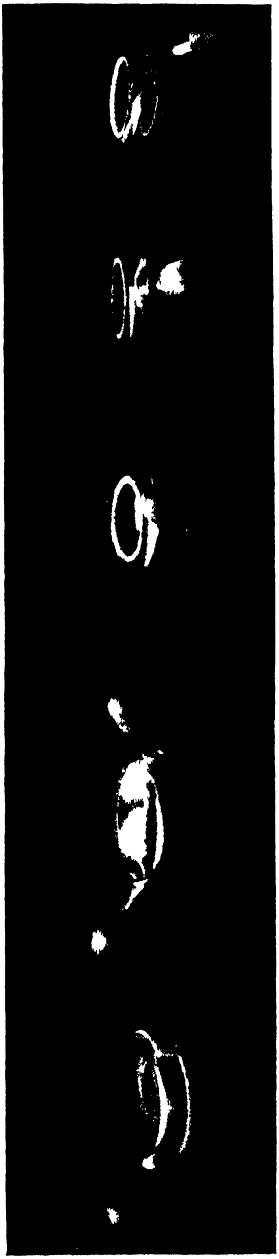


Fig 1

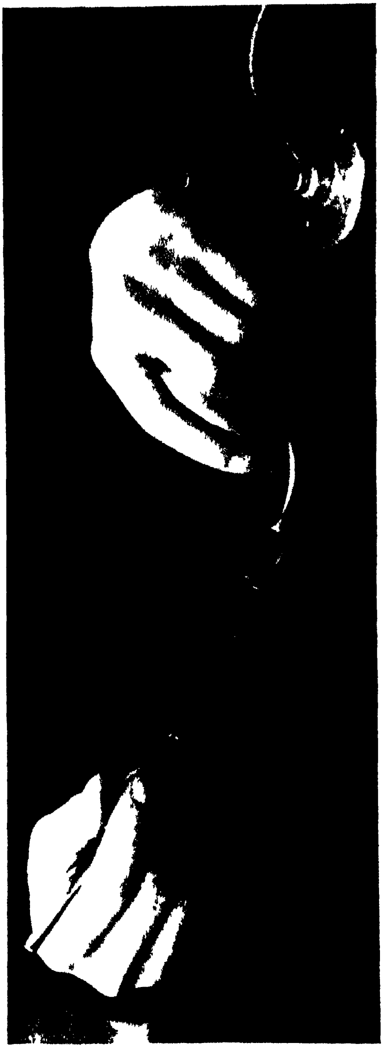
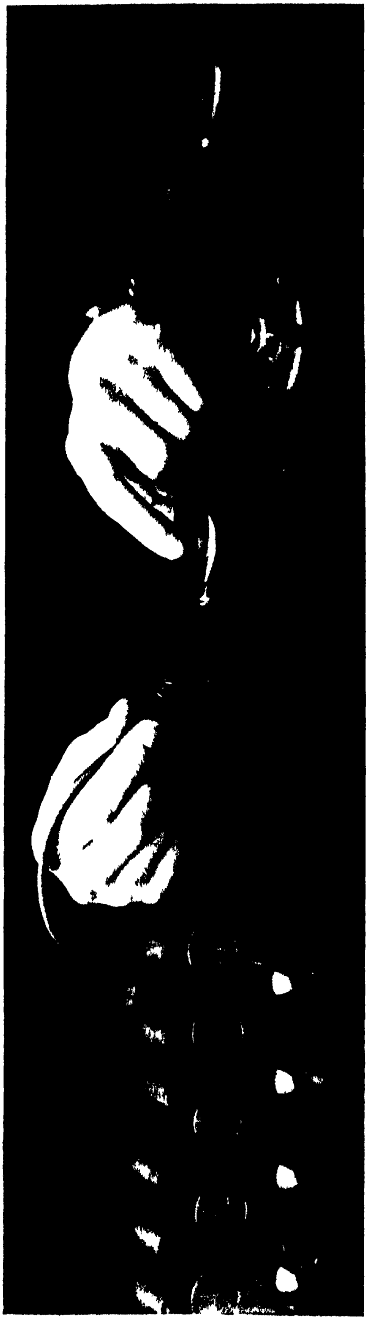
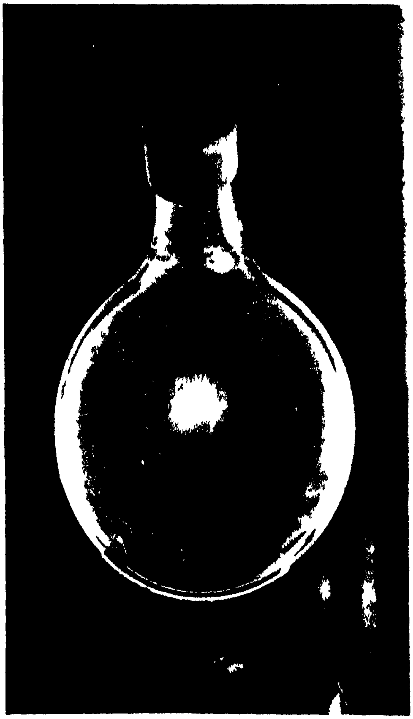


Fig 2

(Carrel Physiological studies of tissues *in vitro*)



FIG



FIG

(Carrel. Physiological studies of tissues *in vitro*.)

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ANTAGONISTIC GROWTH PRINCIPLES OF SERUM AND THEIR RELATION TO OLD AGE.

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I.

INTRODUCTION.

The restraining effect of serum on homologous fibroblasts can be attributed to the antagonistic action of growth-activating and growth-inhibiting substances, the effect of the inhibiting substances always being more pronounced than that of the activating substances.[1,2] The activating substances are thermolabile[3] and accompany the precipitate obtained by bubbling carbon dioxide through diluted serum.[2] They are probably of the same nature as the growth-activating substances contained in embryonic tissue juice[4] and leucocytic extracts or secretions,[5] and may be considered as the product of gland and leucocytic activities.[6] The inhibiting substances resist heat at 65°C.,[3] and remain in the serum with the albumin fraction.[3]

It may be supposed, therefore, that the enhanced restraining action of serum in old age is caused by a decrease of the activating substances, or by an increase of the inhibiting substances, or by both. In order to test this hypothesis, the effect of heating the sera of young and old animals at 65°C., and of precipitating it by carbon dioxide, was studied. If the serum of an old animal contains a lesser amount

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[1] Carrel, A., and Ebeling, A. H , *J. Exp. Med.*, 1921, xxxiv, 599; 1922, xxxvi, 399.

[2] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1923, xxxvii, 653.

[3] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxv, 647.

[4] Carrel, A., *J. Exp. Med.*, 1913, xvii, 14.

[5] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxvi, 365. Carrel, A., *J. Exp. Med.*, 1922, xxxvi, 385.

[6] Carrel, A , and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxvi, 645.

of thermolabile, activating substances than that of a young animal, the inhibiting action of the heated serum of a young animal should be relatively more enhanced than that of an old animal. At the same time, the substances precipitated from the serum of an old animal by carbon dioxide should not stimulate the multiplication of fibroblasts so much as the substances obtained from the serum of a young animal.

II.

Action on Homologous Fibroblasts of the Heated Sera of Young and Old Chickens.

The serum obtained from the plasma of chickens 10 months and 6 years old was heated for 1 hour at 65°C. Its inhibiting action was

TABLE I.

Rate of Growth of Homologous Fibroblasts in Serum, Heated at 65°C. for 1 Hour, from a 10 Month Old Chicken.

Group No.[*]	Culture No.	Rate of growth in serum.		Ratio: $\frac{E}{C}$.	Serum.			
					Original.		Heated.	
		Unheated (*C*).	Heated (*E*).		pH	Refractive index.	pH	Refractive index.
1	31852	3.61	2.15	0.60	8.0	1.3354	7.8	1.3353
2	31853	3.55	2.23	0.63	8.0	1.3354	7.8	1.3353
Average				0.62				

[*] Each group was composed of four experiments.

TABLE II.

Rate of Growth of Homologous Fibroblasts in Serum, Heated at 65°C. for 1 Hour, from a 6 Year Old Chicken.

Group No.[†]	Culture No.	Rate of growth in serum.		Ratio. $\frac{E}{C}$.	Serum.			
					Original.		Heated.	
		Unheated (*C*).	Heated (*E*).		pH	Refractive index.	pH	Refractive index.
1	31854	1.95	1.67	0.86	8.0	1.3370	8.0	1.3370
2	31855	1.95	1.60	0.82	8.0	1.3370	8.0	1.3370
Average				0.84				

[†] Each group was composed of four experiments.

ascertained by comparing the rate of growth of fibroblasts obtained from an 11 year old strain in media containing 50 per cent unheated or heated sera.

In two groups of four experiments, the effects of unheated and heated sera of young and old animals were compared. After being subjected to heat, the sera of the young chickens became 38 per cent more inhibiting, while that of the old chickens became only 16 per cent more inhibiting (Tables I and II).

There was no doubt that the action of heat increases the inhibiting action of the serum of a young animal comparatively more than that of an old animal. Therefore, it may be assumed that in old age the serum contains relatively less thermolabile growth-activating substances than in youth.

TABLE III.

Rate of Growth of Homologous Fibroblasts in Sera from a 10 Month and a 6 Year Old Chicken.

Group No.*	Culture No.	Rate of growth in serum.		Ratio. $\frac{E}{C}$.	Serum.			
					Young.		Old.	
		Young (C).	Old (E).		pH	Refractive index.	pH	Refractive index.
1	31756	3.74	1.96	0.52	8.2	1.3370	8.2	1.3390
2	31808	3.29	2.29	0.70	8.2	1.3370	8.2	1.3390
Average..........		....		0.61				

* Each group was composed of four experiments.

Then it was sought to ascertain whether the heated serum of an old animal is still more inhibiting than that of a young animal, or whether the inhibiting power has become similar. In two groups of four experiments, the action of the serum of a young animal was compared to that of an old animal. The rate of growth of fibroblasts was 39 per cent slower in the serum of the old animal than in that of the young one (Table III). The same experiment was repeated after the sera had been heated, and the inhibiting action of the serum of the old animal was still 24 per cent greater than that of the young animal (Table IV). Then, the increased inhibiting action of serum in old age cannot be considered as due only to the disappearance of thermolabile, activating substances, but also to a more marked effect of the inhibiting principles.

TABLE IV.

Rate of Growth of Homologous Fibroblasts in Sera, Heated at 65°C. for 1 Hour, from a 10 Month and a 6 Year Old Chicken.

Group No.*	Culture No.	Rate of growth in heated serum.		Ratio: $\frac{E}{C}$.	Serum.			
					Young.		Old.	
		Young. (*C*).	Old. (*E*).		pH	Refractive index.	pH	Refractive index.
1	31838	2.18	1.73	0.79	7.8	1.3353	8.0	1.3370
2	31843	2.17	1.59	0.73	7.8	1.3353	8.0	1.3370
Average........................				0.76				

* Each group was composed of four experiments.

TABLE V.

Action of CO_2 Precipitate, from Sera of Young Chickens, on the Rate of Growth of Homologous Fibroblasts.

Group No.*	Culture No.	Age of animal.	Rate of growth in.		Ratio. $\frac{E}{C}$.	Tyrode solution.		Precipitate solution.	
			Tyrode solution (*C*).	Precipitate solution (*E*).		pH	Refractive index.	pH	Refractive index.
		mos.							
1	31689	10	9.34	9.75		7.8	1 3285	7.8	1.3310
2	31702	10	4.84	5.83	1.19	7.8	1 3285	7.8	1.3310
3	31783	10	4.37	5.15		7.8	1.3285	7.8	1.3300
4	31800	10	4.62	5.34	1.17	7.8	1.3285	7.8	1.3300
5	32142	3	4.92	6.12		7.8	1.3279	7.8	1.3291
6	32154	3	4.63	5.54	1.22	7.8	1.3279	7.8	1.3291
		yr.							
7	32103	1	5.01	5 82		7.8	1.3280	7.8	1.3289
8	32114	1	6.58	7.74	1.17	7.8	1.3280	7.8	1.3289
Average............................					1.19				

* Each group averaged from two to four experiments.

III.

Action on Homologous Fibroblasts of the Carbon Dioxide Precipitate Obtained from Sera of Young and Old Animals.

The sera from 3 month, 10 month, 3 year, and 6 year old chickens were precipitated by carbon dioxide according to a technique previously described.[2] The precipitates were dissolved in Tyrode solution and the effect of the respective solutions on homologous fibroblasts was compared to that of Tyrode solution.

TABLE VI.

Action of CO_2 Precipitate, from Sera of Old Chickens, on the Rate of Growth of Homologous Fibroblasts.

Group No.*	Culture No.	Age of animal.	Rate of growth in.		Ratio $\frac{E}{C}$.	Tyrode solution.		Precipitate solution.	
			Tyrode solution (*C*)	Precipitate solution (*E*).		pH	Refractive index.	pH	Refractive index.
		yrs.							
1	31784	3	4.60	4.38		7.8	1.3285	7.8	1.3300
2	31801	3	4.61	4.53	0.97	7.8	1.3285	7.8	1.3300
3	32123	3	5.51	6.06	1.10	7.8	1.3280	7.8	1.3285
4	31690	6	11.05	11.23		7.8	1.3285	8.0	1.3310
5	31703	6	5.33	5.14		7.8	1.3285	8.0	1.3310
6	32102	6	4.86	5.13		7.8	1.3280	7.8	1.3290
7	32113	6	5.87	5.99	1.01	7.8	1.3280	7.8	1.3290
Average.					1.03				

* Each group averaged from two to four experiments.

The growth of fibroblasts in 48 hours was 16 per cent larger in the precipitate solution of the serum of the young animals than in Tyrode solution (Table V), while the activity of the precipitate solution from the serum of the older animals was about identical with that of the Tyrode solution (Table VI). This was a direct proof of the disappearance of the growth-activating substance of serum during the course of life.

TABLE VII.

Action of Serum, after Removal of CO_2 Precipitate, from Young Chickens, on the Rate of Growth of Homologous Fibroblasts.

Group No.*	Culture No.	Age of animal.	Rate of growth in serum.		Ratio: $\frac{E}{C}$.	Serum.			
						Original.		Treated	
			Original (*C*).	Treated (*E*).		pH	Refractive index.	pH	Refractive index.
		mos.							
1	32142	3	3.68	2.91		8.0	1.3338	8.0	1.3322
2	32153	3	3.83	3.08	0.80	8.0	1.3338	8.0	1.3322
3	31719	10	4.96	4.55		8.2	1.3370	8.2	1.3343
4	31732	10	3.59	3.11	0.90	8.2	1.3370	8.2	1.3343
5	31782	10	3.35	2 64		7.2	1 3410	7.2	1.3386
6	31798	10	3.03	2.43	0.81	7.2	1.3410	7.2	1.3386
		yr.							
7	32101	1	2 22	1.85		7.8	1.3360	7.8	1.3348
8	32112	1	3.28	2.84	0.87	7.8	1.3360	7.8	1.3348
Average.....					0.83				

* Each group averaged from three to four experiments.

TABLE VIII.

Action of Serum, after Removal of CO_2 Precipitate, from Old Chickens, on the Rate of Growth of Homologous Fibroblasts.

Group No.*	Culture No.	Age of animal.	Rate of growth in serum.		Ratio: $\frac{E}{C}$.	Serum.			
						Original.		Treated.	
			Original (*C*).	Treated (*E*).		pH	Refractive index.	pH	Refractive index.
		yrs.							
1	32122	3	2.23	2.09	0.94	7.8	1.3408	7.8	1.3372
2	32100	6	1.84	1.71		7.8	1.3361	7.8	1.3350
3	32111	6	2.23	2.09	0.93	7.8	1.3361	7.8	1.3350
Average..............................					0.94				

*Each group was composed of twelve experiments.

IV.

Action on Homologous Fibroblasts of the Serum of Young and Old Animals after the Removal of the Carbon Dioxide Precipitate.

In twenty-eight experiments, divided into eight groups, the effects of the residual sera of the young animals were compared to those of the respective normal sera. It was found that the residual serum had become 17 per cent more inhibiting than the normal serum (Table VII). In three groups of twelve experiments, the action of the residual sera of the old animals was compared to that of the respective normal sera. There was practically no difference in the action of the normal and residual sera within the limits of experimental error (Table VIII).

V.

CONCLUSIONS.

It may be concluded that, under the conditions of the experiments:

1. The inhibiting action on homologous fibroblasts of the heated serum of a young animal increases relatively more than that of an old animal. After it has been heated, the inhibiting action of the serum of the old animal is still greater than that of the young animal.
2. The CO_2 precipitate obtained from the serum of a young animal definitely increases the proliferative activity of homologous fibroblasts, while the CO_2 precipitate of the serum of an old animal has practically no activating power. After the removal of the CO_2 precipitate, the inhibiting action of the serum of young animals is increased, while that of old animals is not modified.
3. The increased inhibiting action of serum on homologous fibroblasts in old age is partly due to the disappearance of the growth-activating substances and to the enhanced activity of the growth-inhibiting principle.

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SURVIVAL AND GROWTH OF FIBROBLASTS IN VITRO.

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(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, June 5, 1923.)

Although the nature of the nitrogenous compounds used by the various tissues in the process of growth is not known, it may be assumed that all cells do not have the same food requirements. Some may receive the nitrogen necessary for the synthesis of new protoplasm from certain amino-acids, peptides, or proteins, while others may require more complex compounds elaborated by other cells. The fibroblasts which, when cultivated in blood serum,[1] multiply temporarily but never increase in mass, proliferate indefinitely as soon as a small amount of embryonic tissue juice[2] is added to the medium. It appears then that the survival or growth of the tissues depends upon the nature of the nitrogenous compounds contained in the medium. It is of interest to know which substances determine the building up of new cells by fibroblasts, and which cannot be utilized for multiplication. Therefore, a study was made of the effect on the proliferation of fibroblasts of egg white, pure egg albumin, egg yolk, and chicken bouillon.

EXPERIMENTAL.

The nutritive value of egg white, egg albumin prepared by the methods of Sörensen and Hopkins, egg yolk, and chicken bouillon, was tested on chicken fibroblasts which were obtained from an 11 year old strain. In most of the experiments, the method of washing and transferring the tissues from medium to medium was used. The control medium was composed of fibrinogen suspension, Tyrode solution with traces of egg yolk, and embryonic tissue juice. In the experiments, the substance to be tested was substituted for an equal amount of Tyrode solution.

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[1] Carrel, A.,.and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 317; 1922, xxxvi, 399.

[2] Carrel, A., *J. Exp. Med.*, 1912, xv, 516. Ebeling, A. H., *J. Exp. Med.*, 1922, xxxv, 755.

Every 48 hours, the growth was measured, and the tissues were washed and transferred into a fresh medium. The same operation was repeated until the tissues died or until their rate of growth became uniform. The effects of Tyrode solution and of the substance under investigation were compared from the point of view of the amount of tissue produced at each passage, and of the duration of life of the cultures. In other experiments, the tissues were studied in the process of uninterrupted growth in a medium which was renewed every 2 or 3 days. The fibroblasts were cultivated in D-5 flasks and imbedded in a coagulum made of 0.5 cc. of plasma and 1.5 cc. of Tyrode solution containing 5 per cent embryo juice. The substance to be tested was poured on the surface of the coagulum. The area of the tissues was measured every 48 hours. The curve expressing the growth of the tissues in the experimental medium was compared to that of the same tissue when the fluid part of the medium was composed of Tyrode solution.

1. Action of Tyrode Solution on Pure Cultures of Fibroblasts.—Rectangular fragments of an 11 year old strain of chicken fibroblasts were placed in a medium composed of chicken fibrinogen 25 per cent, doubly concentrated Tyrode solution 25 per cent, Tyrode solution containing traces of egg yolk and tissue extract 25 per cent, and Tyrode solution 25 per cent.[3] The migration of the fibroblasts began after about 2 hours incubation, and the cells spread rapidly into the coagulum. After 48 hours incubation, the area covered by the new tissue was always large, although the amount of extract contained in the medium was far too small to produce any stimulating effect. The tissues were extirpated from the clot, washed, and transferred to a fresh medium. After each passage the cells proliferated rapidly but, in spite of this apparent activity, the mass of the tissue slowly decreased. A visible, progressive thinning of the central portion of the culture was apparent even on macroscopic observation of the mother fragment. The cells multiplied by no means as rapidly as they migrated. After each passage the area of the fragment not only lessened but the thickness also decreased. As the medium contained no nitrogenous compounds which could be used by the cells, protoplasm could not be synthetized, and the loss of tissue which took place at each passage brought about a rapid decrease in its mass. Under the conditions of the experiment, death occurred generally after from five to six passages, that is, from 8 to 12 days (Text-fig. 1).

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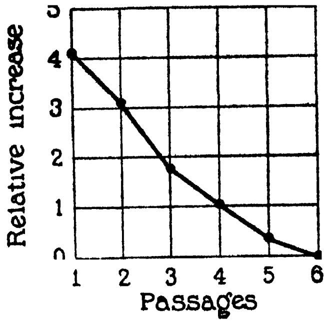
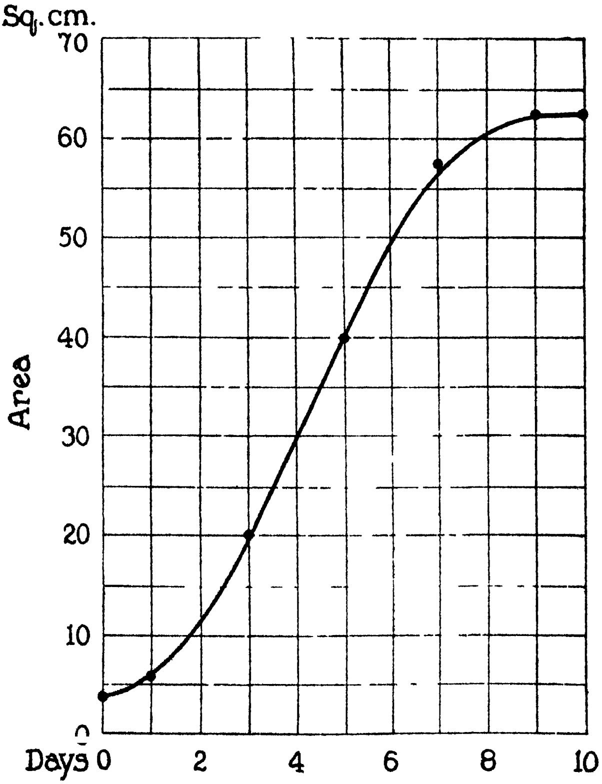
[3] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiii, 641.



TEXT-FIG. 1. Action of Tyrode solution on pure cultures of fibroblasts. The relative increase of pure cultures of fibroblasts during one passage, that is, 48 hours, is plotted in ordinates, and the number of passages in abscissæ.



TEXT-FIG. 2. Action of Tyrode solution on pure cultures of fibroblasts in a condition of uninterrupted growth in a D flask.

Comparative experiments were made with fresh embryonic heart. The fragments survived longer, and showed great activity at the same time that the mass of the tissue slowly decreased.

In other experiments, fragments of a pure strain of fibroblasts or of embryonic heart were placed in D-5 flasks in a medium composed of plasma and Tyrode solution containing 0.5 per cent embryonic tissue juice. In the controls, 1 per cent Tyrode solution was poured into the flask after coagulation had taken place. Every 48 hours, the fluid was aspirated and replaced by the same amount of fresh Tyrode solution. In the experiments, the substance to be tested was substituted for Tyrode solution. The increase in area of the tissue, while in a condition of survival in the non-nutrient medium, was expressed by an S-shaped curve. The growth generally stopped after 7 or 8 days (Text-fig. 2).

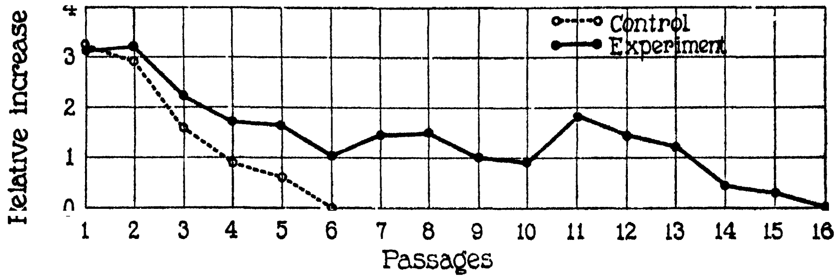
TABLE I.

Action of 25 Per Cent Egg Albumin on Fibroblasts.

		Experiment 1.		Experiment 2.			Experiment 3.		Experiment 4.		Experiment 5.	
		Relative increase.		Relative increase.			Relative increase.		Relative increase.		Relative increase.	
Passage No.	Culture No.	Tyrode solution.	Albumin.	Tyrode solution.	Albumin.	Culture No.	Tyrode solution.	Albumin.	Tyrode solution.	Albumin.	Tyrode solution.	Albumin.
1	446	2.92	2.24	4.04	3.12	459	3.30	3.25	3.40	3.46	3.64	3.83
2	456	2.86	*3.87	3.66	*4.14	470	3.40	*3.35	2.80	*3.46	1.90	*2.50
3	469	2.37	2.86	2.33	2.77	480	1.60	2.40	1.19	1.50	0.88	1.60
4	479	0.81	1.59	1.07	1.49	497	1.23	1.44	1.04	2.92	0.53	1.33
5	496	0.63	1.70	0	1.56	512	0	1.38	0	1.90	0	1.76
6	511	0	1.17		1.06	529		1.12		0.92		1.09
7	528		0.84		0.82	550		2.00		1.87		1.78
8	549		1.84		1.54	567		1.04		1.65		1.38
9	566		1.38		1.13	589		1.06		0.74		0.91
10	588		1.53		0.75	615		0.89		0.80		0.64
11	614		0		0.65	635		3.00		1.83		2.00
12	634				1.50	658		0.93		0.74		2.56
13	657				0	676		1.50		0.80		1.20
14						694		0.18		0.30		1.00
15						711		0		0.30		0
16						724				0		

* Culture divided; ½ discarded.

2. Action of Egg Albumin on Pure Cultures of Fibroblasts.—Solutions of 25 and 35 per cent egg white in Tyrode solution were used. In the cultures containing 25 per cent egg white, the migration of fibroblasts was more active than in the controls containing only Tyrode solution (Table I). The mass of the tissues appeared to increase, and once the tissue fragments had to be divided (Text-fig. 3). But there was no further increase in the bulk of the tissues. The migration remained very active, and the decrease in the size of the tissues was slower than in Tyrode solution. While the control died after five or six passages,



TEXT-FIG. 3. Action of a 25 per cent solution of egg albumin on pure cultures of fibroblasts.

TABLE II.

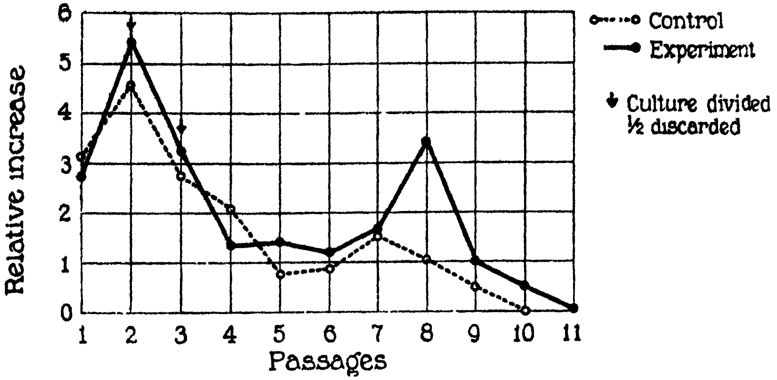
Action of 35 Per Cent Egg Albumin on Fibroblasts.

Passage No.	Culture No.	Experiment 1.		Experiment 2.		Experiment 3.	
		Relative increase.		Relative increase.		Relative increase.	
		Tyrode solution.	Albumin.	Tyrode solution.	Albumin	Tyrode solution.	Albumin.
1	520	3.00	2.87	3.12	2.89	3.31	2.88
2	539	4.74	*4.86	4.00	*5.62	5.00	*5.72
3	551	2.84	*3.47	2.59	*2.74	2.89	*3.84
4	568	2.10	1.32	2.10	1.57	2.00	1.21
5	590	0	1.27	0.78	1.30	0.70	1.69
6	616		2.00		0.78	0.85	0.84
7	636		1.89		1.69	1.50	1.44
8	659		3.27		3.75	1.08	3.25
9	677					0.50	1.00
10	695					0	0.50

* Culture divided; ½ discarded.

the life of the tissues cultivated in egg albumin lasted for fifteen passages, that is, for about 30 days (Text-fig. 3).

Similar experiments were repeated with egg white of a concentration of 35 per cent (Table II and Text-fig. 4). The duration of life was slightly shorter than in the 25 per cent solution. In solutions of higher concentrations than 35 per cent, the growth was very much less.



TEXT-FIG. 4. Action of a 35 per cent solution of egg albumin on pure cultures of fibroblasts.

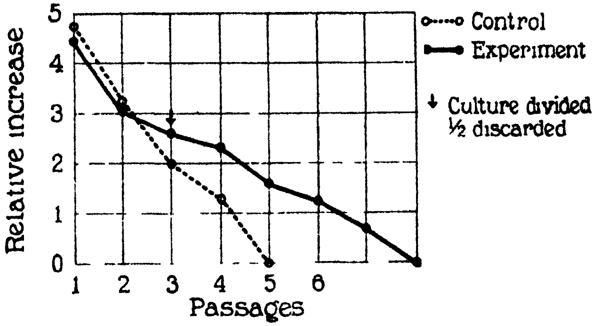
TABLE III.

Action of Egg Yolk on Fibroblasts.

Passage No.	Culture No.	Experiment 1.		Experiment 2.		Experiment 3.	
		Relative increase.		Relative increase		Relative increase.	
		0.25 per cent egg yolk.	2.5 per cent egg yolk	0.25 per cent egg yolk.	2.5 per cent egg yolk.	0.25 per cent egg yolk.	2.5 per cent egg yolk
1	434	5.03	4.06	4.40	4.95	4.89	4.35
2	445	3.38	2.89	3.00	2.74	3.42	3.54
3	455	1.77	2.10	2.10	3.05	2.10	2.64
4	468	1.26	*2.23	0	*2.30	1.50	*2.38
5	481	0	1.56		1.42	0	†1.84
6	499		1.63		0.81		
7	510		0.77		0.60		
8	527		0		0		

*Culture divided; ½ discarded.
†Culture infected; discarded.

In order to ascertain whether the favorable effect of egg white is due to the albumin or to some other substance, the effect on fibroblasts of pure albumin prepared by the methods of Sörensen and Hopkins was ascertained. A solution containing 5 per cent egg albumin prepared by Dr. Fischer according to the Sörensen method was tested on fibroblasts. The medium was made of 1 volume of plasma and 2 volumes of egg albumin. The rate of growth of the fibroblasts was slower than in the control composed of Tyrode solution. Similar experiments were repeated with a solution of egg albumin prepared according to the Hopkins method by Dr. Baker. The rate of growth was also found to be slightly decreased in the albumin solution.

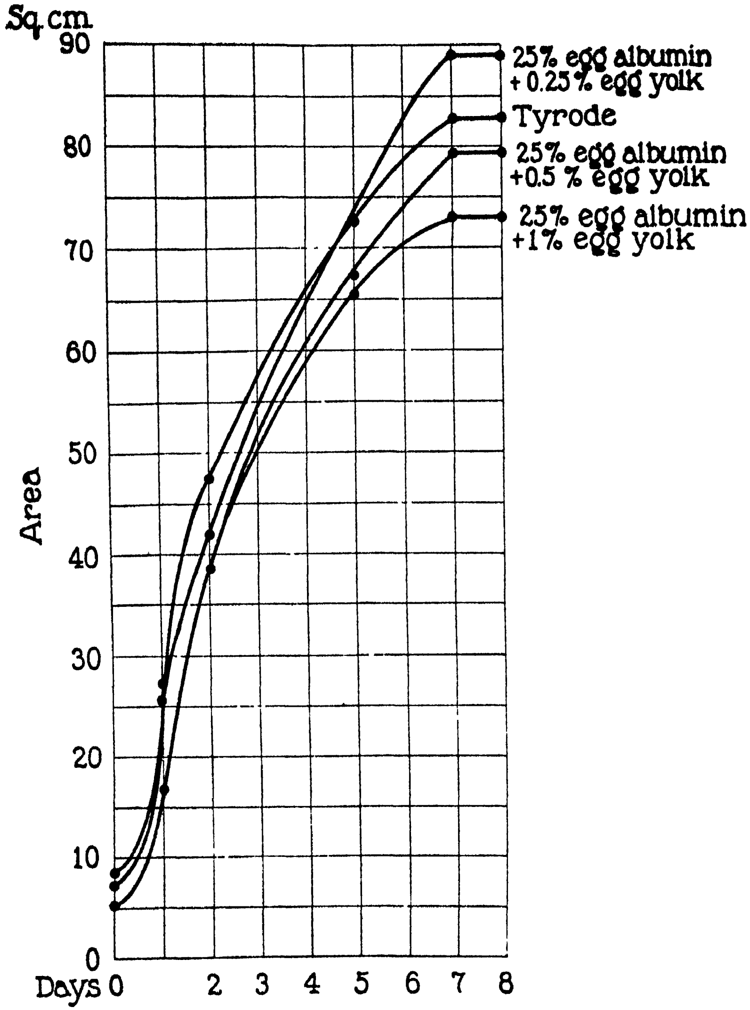


Text-Fig. 5. Action of a 2.5 and a 0.25 per cent solution of egg yolk on pure cultures of fibroblasts.

3. Action of Egg Yolk on Pure Cultures of Fibroblasts.—The effects on pure cultures of fibroblasts of two media containing 0.25 per cent and 2.5 per cent egg yolk were compared (Table III). There was a slight increase in the mass of the tissue and in the duration of life in the medium containing the larger amount of egg yolk. Although the tissues in the controls died after four passages, those in the experiments lived for six or seven passages (Table III and Text-fig. 5).

The effect of higher concentrations of egg yolk on fibroblasts was studied with a different technique. 1 cc. of suspensions of egg yolk in Tyrode solution in concentrations of 5 and 10 per cent respectively was injected into D-5 flasks containing fragments of embryonic heart imbedded in the solid medium. Every 48 hours, the fluid was replaced by a fresh solution. The rate of growth was no greater than in the preceding experiments, showing that fibroblasts are incapable of utilizing egg yolk for growth.

In a final series of experiments, the combined action of egg white and egg yolk was investigated. The fluid medium of D-5 flasks contained 25 per cent egg white and 1, 0.5, and 0.25 per cent, respectively, egg yolk. The rate of growth and the duration of life of the tissues were not increased (Text-fig. 6).



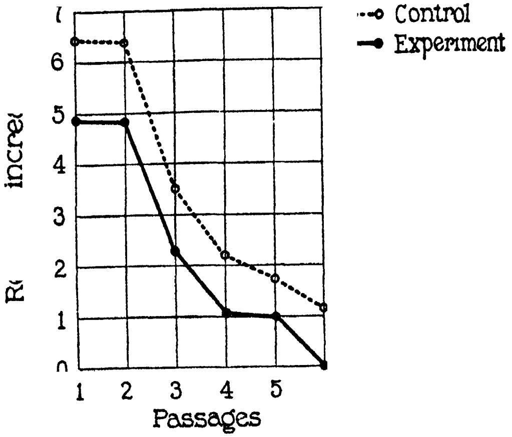
TEXT-FIG. 6. Simultaneous action of egg yolk and egg white on pure cultures of fibroblasts in continuous growth in a D flask.

4. Action of Chicken Bouillon on Pure Cultures of Fibroblasts.—Bouillon was prepared from chicken tissues and added to the medium in concentrations varying from 10 to 20 per cent. In the cultures containing 20 per cent bouillon, the amount of growth was markedly decreased and also the duration of life (Table IV and Text-fig. 7). Concentrations below 10 per cent, such as used by Lewis and Lewis in some of their experiments with nutrient agar,[4] had no retarding effect. The growth of the cultures in bouillon was about equal to that of the

TABLE IV.

Action of 20 Per Cent Bouillon on Fibroblasts.

Passage No.	Culture No.	Experiment 1.		Experiment 2.		Experiment 3.		Experiment 4.	
		Relative increase.		Relative increase.		Relative increase.		Relative increase.	
		Tyrode solution	Bouillon.	Tyrode solution.	Bouillon.	Tyrode solution.	Bouillon.	Tyrode solution.	Bouillon.
1	746	6.94	4.41	6.47	5.13	7.54	4.77	5.00	5.31
2	755	6.30	5.50	6.34	4.34	6.35	4.95	6.60	4.78
3	771	3.25	2.53	2.97	2.22	3.24	2.54	2.75	1.89
4	779	2.85	0.74	1.90	1.80	1.96	1.54	1.94	0.23
5	796	1.63	0	1.67	0.89	1.65	1.20	1.89	0
6				1.16	0	1.21	0		



TEXT-FIG. 7. Action of 20 per cent bouillon on pure cultures of fibroblasts.

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[4] Lewis, M. R., and Lewis, W. H., *Bull. Johns Hopkins Hosp.*, 1911, xxii, 126.

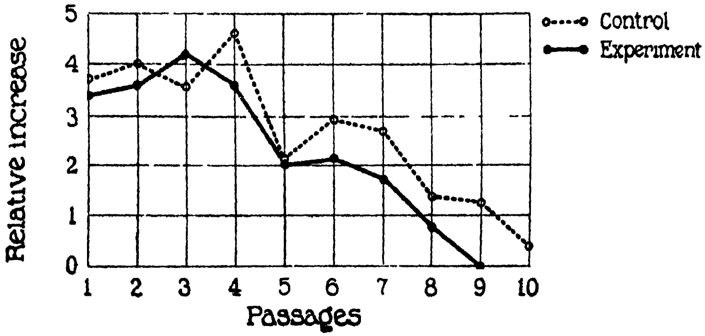
cultures in Tyrode solution. The duration of life was not affected (Table V and Text-fig. 8).

In other experiments, a bouillon was made of embryonic tissue juice, heated in a sealed tube at 100°C. for 1 hour. It had a slightly retarding action on the multiplication of fibroblasts.

TABLE V.

Action of 10 Per Cent Bouillon on Fibroblasts.

Passage No.	Culture No.	Experiment 1.		Experiment 2		Experiment 3.		Experiment 4.	
		Relative increase.		Relative increase		Relative increase.		Relative increase.	
		Tyrode solution.	Bouillon	Tyrode solution	Bouillon.	Tyrode solution.	Bouillon.	Tyrode solution.	Bouillon.
1	672	3.40	4.00	3.89	3.10	3.60	3.09	3.91	3.38
2	685	3.23	3.95	5.00	3.00	4.00	3.50	3.95	3.60
3	701	2.94	4.00	3.50	3.93	3.66	4.46	4.00	4.45
4	718	4.75	3.00	4.00	3.50	4.64	2.66	4.64	5.10
5	731	2.58	2.34	2.16	3.00	1.71	1.56	1.60	1.28
6	745	3.15	2.00	2.61	2.13	2.85	2.00	3.00	2.36
7	754	2.57	0	2.87	0	2.25	1.53	2.86	2.00
8	770	1.33		1.70		2.00	0	1.10	0.75
9								1.24	0
10								0.39	



TEXT-FIG. 8. Action of 10 per cent bouillon on pure cultures of fibroblasts.

DISCUSSION AND CONCLUSIONS.

When multiplying actively in serum, fibroblasts are in a condition of survival, not of cultivation, for they do not synthetize protoplasm from substances contained in the medium. They are able to utilize only the nitrogenous material stored in the tissue itself. The distinction between survival of tissue and active growth is an important one. Innumerable errors in the interpretation of the experimental results have been made during the past years on account of this lack. An active proliferation of fibroblasts taking place during several days in a medium containing a given substance does not mean that this substance is used by the cells, but indicates only that residual cell proliferation is unopposed by a toxic substance. It is not possible to ascertain whether a medium is nutrient or not in the sense that it supports growth unless the mass of the tissues is shown to increase. When the proliferating fragment can be halved again and again, and each half reaches the size of the original whole in 48 hours, it is evident that new protoplasm must have been synthetized from the medium. The experiments described above show that egg white, egg yolk, pure egg albumin, egg white mixed with egg yolk, and bouillon are not utilized by fibroblasts for multiplication. This fact renders more plausible the hypothesis advanced previously that for proliferation the fibroblasts require some nitrogenous substances elaborated by other cells, which are present in embryonic tissue juices.

It may be concluded that, under the conditions of the experiments:

1. The duration of life of pure cultures of fibroblasts in a nitrogen-free medium is about 8 days.

2. Egg white and egg yolk, isolated or together, slightly increase the activity of the fibroblasts, but this effect is temporary and the tissues ultimately die. Pure egg albumin and bouillon are not utilizable by fibroblasts for multiplication.

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ACTION ON FIBROBLASTS OF EXTRACTS OF HOMOLOGOUS AND HETEROLOGOUS TISSUES.

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(Received for publication, June 5, 1923.)

Fibroblasts do not multiply in blood serum[1] after the food material stored in the original tissue is exhausted.[2] Neither can they obtain the nitrogen necessary for their proliferation from chicken bouillon, egg white, egg yolk, nor pure egg abumin.[3] But they live and multiply indefinitely when the medium contains a small amount of embryonic tissue juice.[4] It is, therefore, probable that they require for the building up of protoplasm certain nitrogenous compounds which are synthetized within the organism by other cells. It is known that leucocytes contain such growth-promoting substances.[5] Extracts of muscle and gland tissues of adult animals also possess the power of stimulating the rate of growth of homologous fibroblasts *in vitro*.[6] The juice of heterologous tissues, such as guinea pig tissue, may determine the proliferation of chicken fibroblasts, although the life of these cells in such a medium is not permanent.[7] It seemed that heterologous as well as adult tissues contain certain substances which permit a pure culture of fibroblasts to increase in mass, at least for a time. The aim of the experiments described in this article was to determine as accurately as possible, with the techniques at our disposal, the action of homologous adult tissue extracts and of heterologous embryonic juices and adult tissue extracts on the rate of growth of chicken fibroblasts and on the duration of their life *in vitro*.

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[1] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 317; 1923, xxxvii, 759.

[2] Lewis, M. R., and Lewis, W. H., *Anat. Rec.*, 1911, v, 277. Ingebrigtsen, R., *J. Exp. Med.*, 1912, xvi, 421. Burrows, M. T., *Anat. Rec.*, 1916–17, xi, 335. Burrows, M. T., and Neymann, C. A., *J. Exp. Med.*, 1917, xxv, 93. Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 317.

[3] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1923, xxxviii, 487.

[4] Carrel, A., *J. Exp. Med.*, 1912, xv, 516. Ebeling, A. H., *J. Exp. Med.*, 1922, xxxv, 755.

[5] Carrel, A., *J. Exp. Med.*, 1922, xxxvi, 385. Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxvi, 645.

[6] Carrel, A., *J. Exp. Med.*, 1913, xvii, 14.

[7] Carrel, A., unpublished experiments.

EXPERIMENTAL.

Young embryos of chickens, mice, guinea pigs, and rabbits were finely pulped and centrifuged. The supernatant juice was diluted with approximately its volume of Tyrode solution. Muscle and organs of adult chickens, mice, guinea pigs, and rabbits were sliced in a Latapie apparatus, and to the pulp was added its volume of Tyrode solution. After a few hours, the mixture was centrifuged and the supernatant fluid removed.

The juices and extracts were kept in a refrigerator and their H ion concentration was determined daily by the colorimetric method. The pH was about 7.8, and after a few days it became lower, especially in extracts of adult tissues. It was kept constant by the addition of small amounts of sodium hydrate. The juices and extracts were always used within a week after they had been prepared.

The action of the juices and extracts was tested on an 11 year old strain of fibroblasts. The stock cultures were divided into two equal parts. One of the fragments was placed in a medium composed of 1 volume of plasma and 2 volumes of Tyrode solution containing 0.02 of chick embryo juice, and used as a control, while the other fragment was placed in 1 volume of plasma and 2 volumes of tissue extract or tissue juice. The fragments were traced in a projectoscope after 1 and 48 hours and the relative increase was measured by the usual technique.[8] If, after 48 hours, the tissue fragments had doubled in size, they were divided into two equal parts, one half being discarded, and the remaining half cultivated in the same medium. When no marked increase had occurred, the fragments were merely washed and transferred to a fresh medium. The figures on relative increase of the fragments, their absolute dimensions at the beginning of each passage, and the number of passages during which they lived, made it possible to ascertain the action of the various juices and extracts on cell proliferation. The data for each experiment were tabulated, but to save space the tables have been omitted. For each group of experiments, a chart shows the variations in the relative increase of the tissues in function of the time, which is expressed by the number of passages.

1. Action of Fresh Chick Embryo Juice and of the Same Juice Preserved in Cold Storage on the Duration of Life of Homologous

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[8] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.

Fibroblasts.—Before studying the action of the various homologous and heterologous juices on the growth of chicken fibroblasts, it was necessary to ascertain in more precise manner than has been done heretofore the influence of the spontaneous modifications undergone by the juices in function of time. It is well known that the increase in the H ion concentration prevents the stimulating action of the juice on cell proliferation,[9] and that this increase occurs spontaneously. But as it is also possible that some other deterioration takes place, even when the pH does not vary, the action of fresh juices was compared with that of juices kept in cold storage at a constant pH. In the following experiments, fresh juice and juice kept in cold storage were compared. When the juice had been kept in the refrigerator for more than 1 week, its action became weaker than that of the fresh extract. The tissues which had been cultivated in the juice kept for 34 days in the refrigerator died after seventeen passages, while those cultivated in fresh extract continued doubling in size every 48 hours (Text-fig. 1). It was not possible to determine whether the cause of death was intoxication or starvation. This experiment showed that, when the H ion concentration was kept constant by the addition of sodium hydrate, some changes occurred in the embryonic tissue juice which rendered it unsuitable for maintaining fibroblasts in a condition of indefinite growth. But this effect could hardly have been detected if the tissues had not been in contact for several passages with the juice. Tissue juices and extracts should be used, therefore, when freshly prepared.

2. Action of Extracts of Adult Homologous Tissues on the Growth and Duration of Life of Fibroblasts.—The extracts made from muscle brought about an increase in the mass of the tissues during the first passage. In one group of three experiments, the tissues could be divided into two parts at the third passage (Text-fig. 2), and apparently the muscle tissue extract had an action similar to that of embryonic tissue juice. However, it was observed that the cells contained more fat granules than do normal cultures. After either four or five passages, the tissues died. The controls in Tyrode

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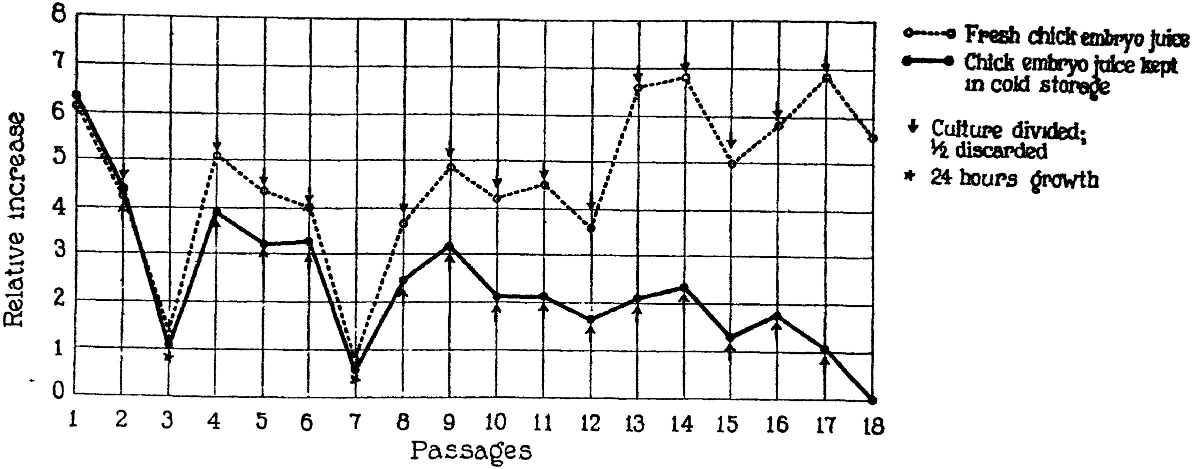
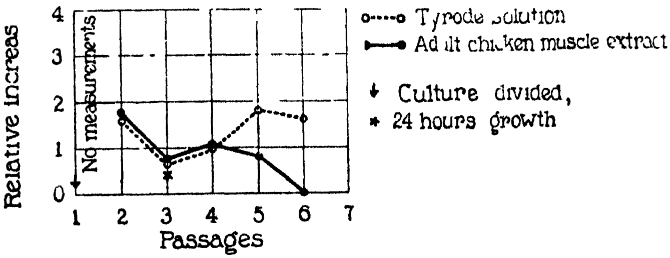
[9] Fischer, A., *J. Exp. Med.*, 1921, xxxiv, 447.



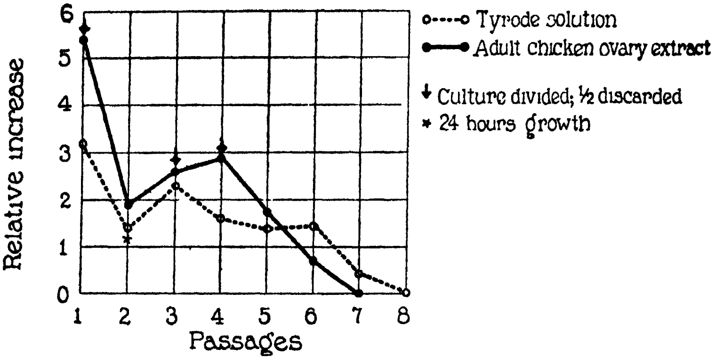
Text-Fig. 1. Action of fresh chick embryo juice and of chick embryo juice kept in cold storage on the rate of growth of homologous fibroblasts.

solution did not increase in size to such an extent that they could be divided. They were still living after six passages (Text-fig. 2).

In one group of three experiments, extracts of ovary also had a stimulating action. The mass of the tissues increased, but not permanently. The cultures were large enough to be divided after the first, third, and fourth passages. Death occurred at the seventh passage. The controls did not increase in mass and could not be divided, but they survived seven passages (Text-fig. 3). Thus, it appeared that the extracts of adult tissues were not capable of maintaining indefinitely the life of the fibroblasts *in vitro*, as fresh embryonic juice does.

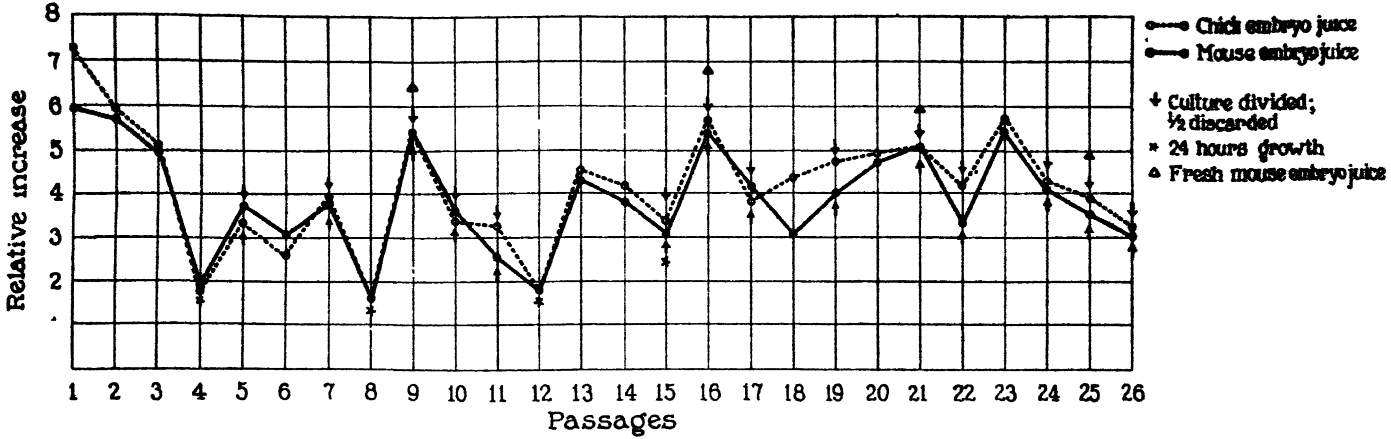


TEXT-FIG. 2. Action of adult chicken muscle extract on the duration of life of homologous fibroblasts.



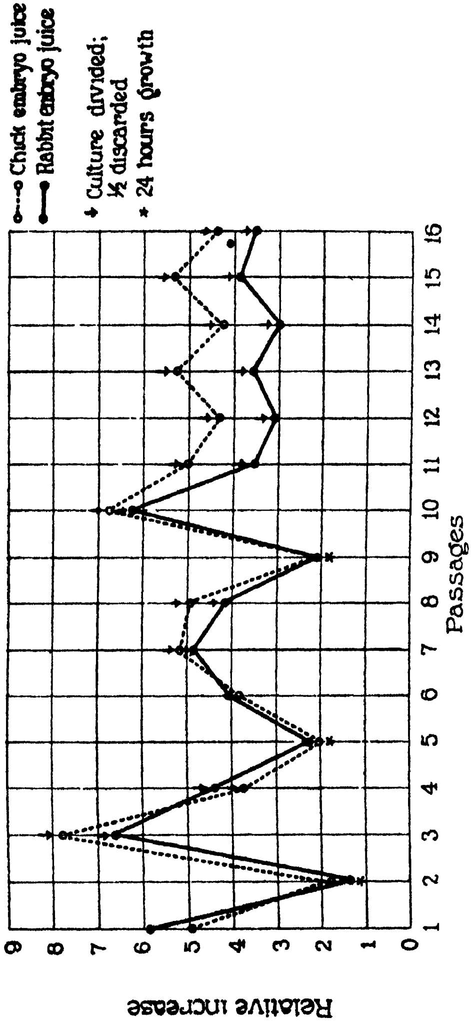
TEXT-FIG. 3. Action of adult chicken ovary extract on the duration of life of homologous fibroblasts.

3. Action of Heterologous Embryonic Tissue on the Growth and Duration of Life of Fibroblasts.—In one group of three experiments,



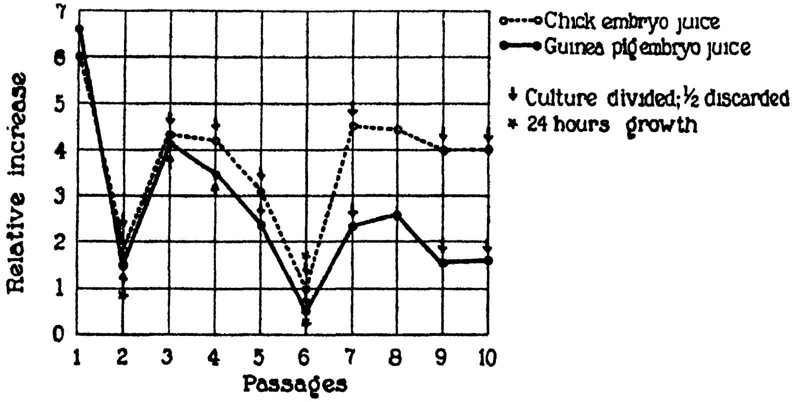
TEXT-FIG. 4. Action of mouse embryo juice on the rate of growth of chicken fibroblasts.

the action of the juice of mouse embryos was tested on chicken fibroblasts, the controls being cultivated in chick embryo juice. The supply of mouse embryo juice was renewed after 12, 26, 36, and 44 days. Text-fig. 4 shows that the connective tissue fragments doubled in mass in 48 hours in mouse embryo juice, as well as in chick embryo juice. After twenty-six passages, the rates of growth were still practically the same in both substances and the

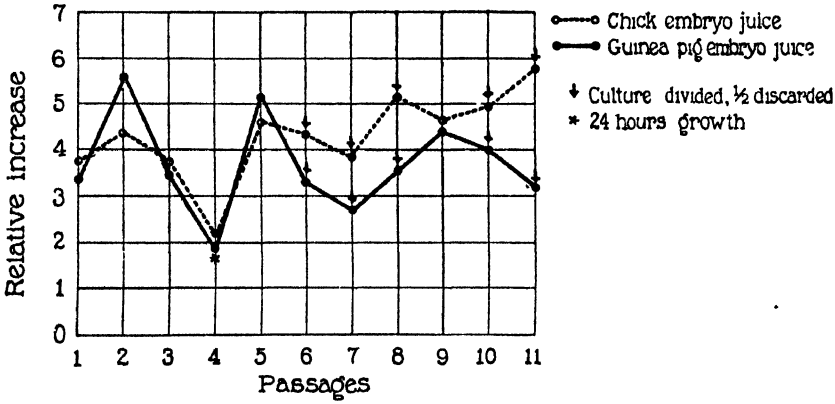


TEXT-FIG. 5. Action of rabbit embryo juice on the rate of growth of chicken fibroblasts experiment was discontinued. No evidence had been obtained that the tissues would die sooner in mouse embryo juice than in chick embryo juice.

In one group of three experiments, rabbit embryo juice acted on chicken fibroblasts in the same manner as chick embryo juice. The tissues increased markedly in size and no difference could be observed between fibroblasts nourished on chick embryo juice and rabbit embryo juice. Every 48 hours, the tissues doubled in size. This experiment was continued for 29 days. The curve shows that the

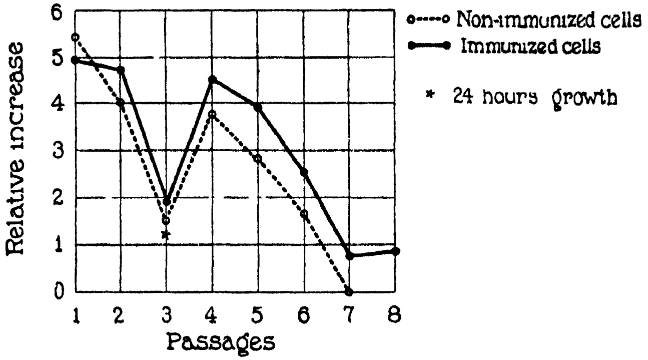


TEXT-FIG. 6. Action of guinea pig embryo juice on the rate of growth of chicken fibroblasts.

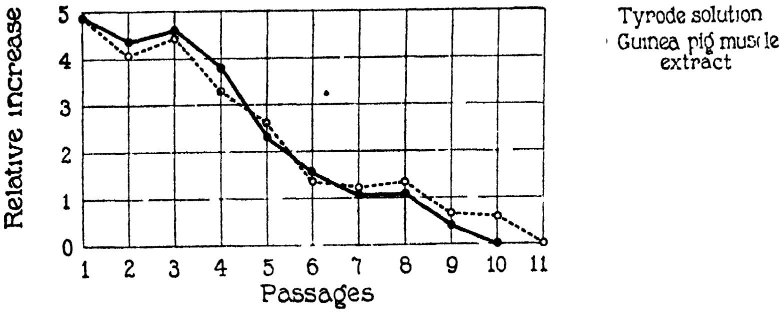


TEXT-FIG. 7. Action of guinea pig embryo juice on the rate of growth of chicken fibroblasts.

rates of growth of the fibroblasts in both juices were in general similar (Text-fig. 5). The differences observed after the 19th day were due probably to the fact that the supply of rabbit embryo juice had not been renewed. A similar slackening in the growth was observed when homologous tissue juice was used under the same conditions.



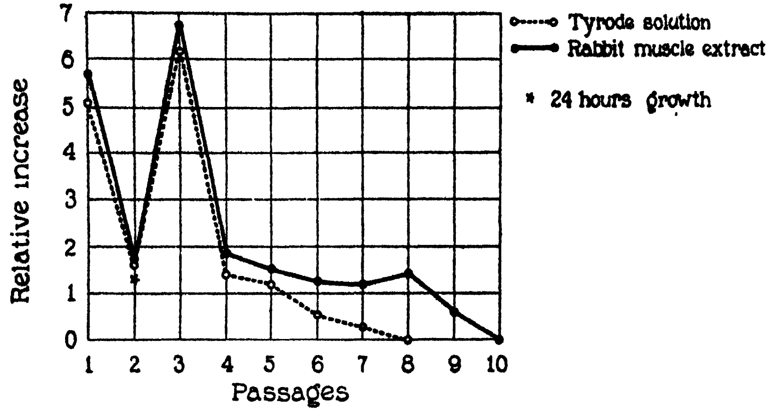
TEXT-FIG. 8. Action of rabbit serum on the rate of growth of chicken fibroblasts cultivated previously in chicken and rabbit embryo juices.



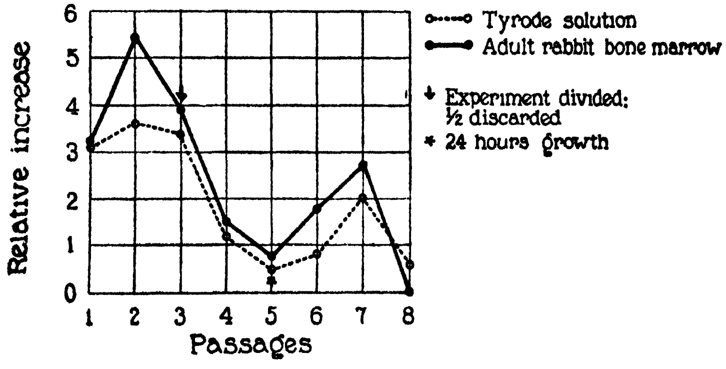
TEXT-FIG. 9. Action of adult guinea pig muscle extract on the duration of life of chicken fibroblasts.

In two groups of three and four experiments respectively, the action of guinea pig embryo juice was studied in the same manner. In a first series of experiments, the tissues doubled in mass every 2 days in both media. After seven passages, growth was more extensive in the chick embryo juice because the supply of guinea pig juice had not been renewed (Text-fig. 6). In a second series of experiments, after 20 days the rate of growth of chicken tissue fed on guinea pig juice was the same as that of the control (Text-fig. 7).

It is evident that protoplasm can be synthetized by chicken fibroblasts nourished on guinea pig, mouse, and rabbit embryonic tissues.

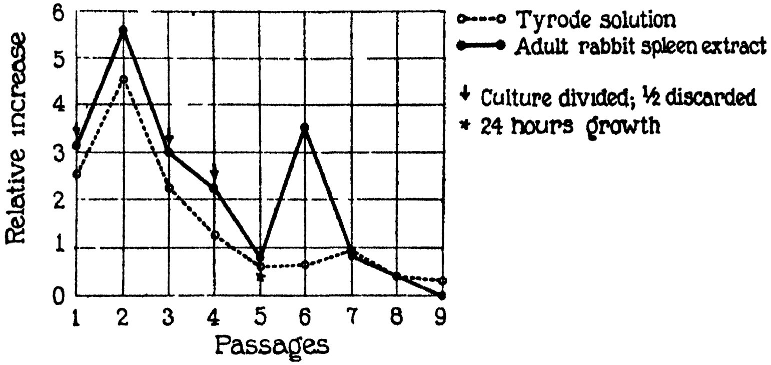


Text-Fig. 10. Action of adult rabbit muscle extract on the duration of life of chicken fibroblasts.

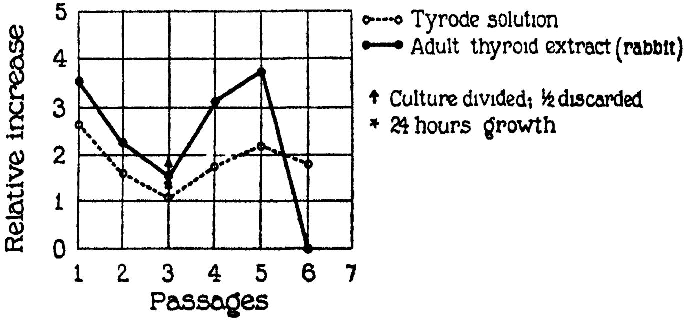


Text-Fig. 11. Action of adult rabbit bone marrow extract on the duration of life of chicken fibroblasts.

No differences traceable to variations in the media could be observed either in the rate of migration or the amount of tissue produced. Nevertheless, in one group of three experiments, an attempt was made to investigate whether chicken fibroblasts fed on rabbit juices were modified in their specificity. Strains of chicken fibroblasts derived respectively from cultures maintained on chicken and rabbit embryo substances were cultivated in rabbit serum. The retarding action of rabbit serum was less marked on the fibroblasts grown in rabbit tissue juice than on the normal fibroblasts (Text-fig. 8). The fibroblasts previously nourished on rabbit tissue juice underwent but a slight change. This effect may have been due to the fact that the specificity of the chicken fibroblasts so nourished had decreased, or that they had become immunized against rabbit specific substances.



TEXT-FIG. 12. Action of adult rabbit spleen extract on the duration of life of chicken fibroblasts.



TEXT-FIG. 13. Action of adult rabbit thyroid extract on the duration of life of chicken fibroblasts.

4. Action of Heterologous Adult Tissue Extract on the Growth and Duration of Life of Fibroblasts.—In one group of four experiments, chicken fibroblasts cultivated in extract of adult guinea pig muscle did not increase in mass, and died sooner than the controls in Tyrode solution (Text-fig. 9).

In one group of three experiments, the extract of adult rabbit muscle had a similar action. The fibroblasts died 4 days sooner in muscle extract than in Tyrode solution (Text-fig. 10).

In one group of three experiments, rabbit bone marrow caused the fibroblasts to double in mass at the third passage. But both the experiment and control tissues died at the eighth passage (Text-fig. 11).

In one group of four experiments, rabbit spleen extract increased the mass of the tissues to the same extent, but the duration of life was no longer than in the controls (Text-fig. 12).

In one group of three experiments, extract of thyroid gland produced some increase in the mass of the tissues. Death occurred suddenly at the sixth passage (Text-fig. 13).

SUMMARY.

Extracts of homologous adult tissues determine an increase in the mass of pure cultures of chicken fibroblasts nourished thereon comparable to that resulting from embryonic tissue juice. But the effect of these extracts differs markedly from that of the latter, since cell multiplication does not continue indefinitely. After a few passages, the fibroblasts cultivated in adult tissue extracts grew more slowly than in Tyrode solution. The cytoplasm became dark and filled with fat granules, and death followed. It is possible that the tissues of adult animals contain, as does the serum, substances which are toxic for the homologous cells, and which progressively overcome the effect of the growth-activating substances. The effect of heterologous adult tissue extracts did not differ markedly from that of homologous tissues. The chicken connective tissue increased slightly in mass, but died sooner than the controls in Tyrode solution.

By contrast, tissue juices derived from the embryos of mice, guinea pigs, and rabbits acted on chicken fibroblasts in the same manner as chick embryo juices. The increase in mass of the cultures was regular and rapid. They doubled in size every 48 or 72 hours, and the

rate of growth did not decrease after 30 days. It appears that embryonic tissue juices are not necessarily toxic for heterologous fibroblasts, and that they can be used in the building up of protoplasm in the tissues of a different species. In experiments made long ago,[6] the action of tissue juice was described as being specific. The premature death of the fibroblasts cultivated in heterologous juices at that time would now appear to have been due to spontaneous changes in the pH and the deterioration that even normal chick embryo juice at a pH of 7.8 undergoes spontaneously. In the recent experiments, when freshly prepared homologous and heterologous juices were used, their action on chicken fibroblasts in pure culture was identical. However, the fibroblasts produced in cultures nourished by rabbit juice grew better when transferred to rabbit serum than did ordinary chicken fibroblasts. It has not been determined as yet whether this effect is due to an immunization of the fibroblasts against rabbit humors, or to some decrease in the specificity eventuating in cells intermediate between rabbit and chicken fibroblasts.

It may be concluded that, under the conditions of the experiments:

1. Pure cultures of chicken fibroblasts increase in mass under the influence of extracts of adult homologous tissues. But they ultimately die while the fibroblasts cultivated in embryonic tissue juices live indefinitely.
2. The increase in mass of chicken fibroblasts cultivated in the juices of mouse, guinea pig, rabbit, and chick embryos is about identical.
3. Chicken fibroblasts produced in cultures nourished by rabbit embryonic tissue juice are less sensitive to the inhibiting action of rabbit serum than ordinary chicken fibroblasts.
4. Cultures of chicken fibroblasts in extracts of adult tissues of mice, guinea pigs, and rabbits increase slightly in mass, but the increase is temporary and death occurs after a few passages.

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ACTION OF SERUM ON LYMPHOCYTES IN VITRO.

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(From the Laboratories of The Rockefeller Institute for Medical Research.)

Plates 32 and 33.

(Received for publication, June 5, 1923.)

Fibroblasts do not obtain from serum,[1] egg white, pure egg albumin, bouillon,[2] or amino acids,[3] the nitrogen necessary for their nutrition. When cultivated with these substances, they fail to multiply even in the presence of egg yolk which contains the necessary accessory factors.[3] It appears that they require for their proliferation certain special substances synthetized by embryonic tissue.[1] There is evidence for the view that, *in vivo*, they obtain these elements from the white blood corpuscles which possess the property of manufacturing and secreting them.[4] Should this conjecture be true, leucocytes must be capable of transforming some of the serum constituents into the more complex compounds necessary for the nutrition of the fibroblasts and epithelial cells. Therefore, an attempt was made to ascertain whether lymphocytes and large mononuclear cells multiply in a medium containing serum as the sole source of nitrogen, and set free substances determining the proliferation of a pure strain of fibroblasts.

EXPERIMENTAL.

1. Action of Serum on the Multiplication of Lymphocytes in Vitro.— Fragments of embryo spleen or of a coagulum containing white blood corpuscles were imbedded in a medium consisting of 1 cc. of chicken plasma, 1.5 cc. of Tyrode solution containing 10 per cent embryonic tissue juice, and 1.5 cc. of Tyrode solution, according to a technique previously described.[5] After 2 days incubation, 1 cc. of serum diluted with its volume of Tyrode solution was introduced into the flask. After 3 or 4 days, the fluid was aspirated and replaced by 1 cc. of pure Tyrode solution for 24 hours when diluted serum was again placed in the flask.

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[1] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 317; 1923, xxxvii, 759.

[2] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1923, xxxviii, 487.

[3] Carrel, A., and Ebeling, A. H., unpublished experiments, 1923.

[4] Carrel, A., *J. Exp. Med.*, 1922, xxxvi, 385. Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxvi, 365, 645.

After a few hours, a great many ameboid cells wandered from the original fragment and invaded the culture medium. The polymorphonuclear cells disappeared after a short time, and lymphocytes and large mononuclear cells migrated through the coagulum as individual units without any tendency to form a tissue. As the experiments were performed early in the development of the special culture method utilized, several contaminations or other accidents occurred, and only a few cultures were successfully observed for more than a month.

Two cultures were prepared in D flasks, 5 cm. in diameter, which contained one and three fragments of spleen about 1 sq. mm. in area. The migration and multiplication of the cells proceeded slowly. The rate of multiplication increased temporarily each time fresh serum was added to the medium. The wandering cells invaded the medium at first on one plane in an orderly fashion, each cell being separated from the neighboring cells by practically regular intervals. Later, they grew on three or four planes (Fig. 1), but without any contact or tendency to tissue formation. They accumulated around a few foreign bodies which were in the medium, as they generally do *in vivo*. After 30 days, practically the entire medium was invaded by the wandering cells. They covered a circle about 45 mm. in diameter, that is, an area of about 2,000 sq. mm. As the surface area of the original fragment of spleen was about 1 sq. mm., it was evident that the lymphocytes must have multiplied. In other cultures, small islands of lymphocytes appeared at a distance from the original fragment of spleen. The growth of these islands could be readily observed, showing that the spreading of the cells is due to the multiplication, and not merely to the migration, of cells contained in the original fragment of spleen. There is no doubt that the number of lymphocytes increased immensely during their cultivation in serum.

[5] Carrel, A., *J. Exp. Med.*, 1923, xxxviii, 407.

One of the cultures died of bacterial contamination after 36 days. As soon as the infection was detected, the fluid part of the medium was removed, the cultures were washed in Tyrode solution, fixed in formalin, and stained with eosin-azure. Many of the cells were dead and had become round when the culture was fixed. No polymorphonuclear elements were seen. All the cells appeared to be lymphocytes and large mononuclear cells (Fig. 2), with fibroblasts at the site of the original fragment.

In the second culture, the coagulum became detached from the bottom of the flask after the 34th day. The cells were in full activity. Some of the fragments of the coagulum were fixed and stained. The cells proved to be mononuclears and were large and active (Figs. 3 and 4).

Similar experiments were made with leucocytes obtained from blood. After chicken blood had been centrifuged for 10 minutes, a few drops of a dilute solution of embryonic tissue juice were placed on the corpuscles. Coagulation occurred, and fragments from the film containing the white blood corpuscles were cultivated in D-5 flasks. The fluid part of the medium was composed of Tyrode solution containing 30 per cent normal serum, and was renewed every 3 or 4 days. After 21 days, cells resembling lymphocytes had invaded practically the entire culture medium. They stopped at about 0.5 cm. from the edge of the flask, and died 10 days later.

2. *Action of Lymphocytes and Large Mononuclear Cells on Fibroblasts Contained in a Medium Composed of Serum.*—Previous experiments have shown that when a fragment of spleen is placed at a short distance from a culture of fibroblasts, the rate of multiplication of the fibroblasts is markedly increased,[6] owing to the secretion of growth-promoting substances by the leucocytes. It was found also that leucocytes do not live more than 10 days in Tyrode solution.[6] This means that in a non-nutrient medium, they exhausted their reserves in from 7 to 10 days. It follows that, if leucocytes cultivated in blood serum for more than 10 days are able to increase in rate of migration and multiplication of fibroblasts, it would indicate that the material used by them in the elaboration of the growth-promoting substances must come from the serum.

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[6] Carrel, A., unpublished experiments, 1922–23.

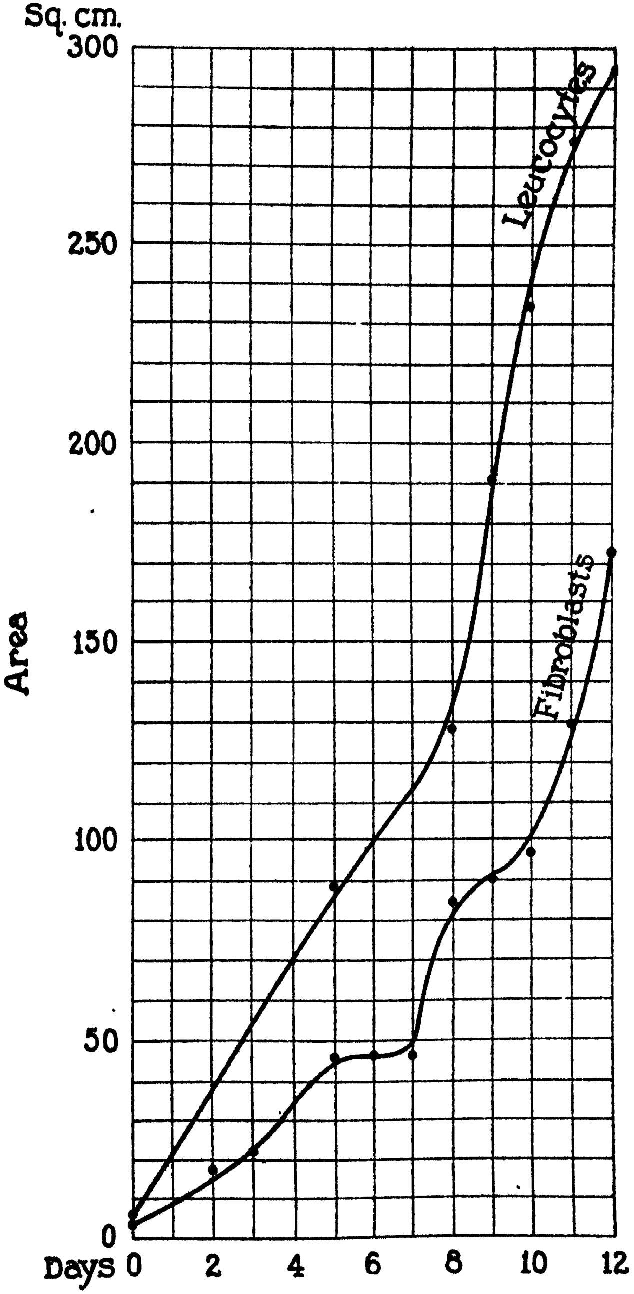
In a D-5 flask, a fragment of spleen was placed 12 mm. from a fragment of a pure culture of fibroblasts. As the fluid part of the medium was composed of pure serum, the fibroblasts stopped proliferating after 5 days. Meanwhile, the leucocytes slowly invaded the medium and reached the neighborhood of the colony of fibroblasts. By the 9th day, the fibroblasts had become very large and their cytoplasm was dark and loaded with fat granules. Serum was now added to the medium and the lymphocytes proliferated very actively and some of them reached the fibroblasts. During the following days, the appearance of the fibroblast's changed. They became slender and elongated at the same time that the cytoplasm decreased in volume, and they again began to proliferate actively, as indicated on the curve (Table I and Text-fig. 1). The number of leucocytes increased greatly and, on the, 12th day, a great many began to die, owing probably to the accumulation of too large a number of cells in a comparatively small area. The fibroblasts were then quite refreshed and in full activity (Text-fig. 1).

TABLE I.

Action of Lymphocytes on Fibroblasts in a Medium Composed of Serum.

Date.	Culture No.	Area of		Composition of fluid medium.
		Lymphocytes.	Fibroblasts.	
1923				
Feb. 19	2667-3	5.1	4.6	0
“ 20				Serum 1 cc.
“ 21			18.1	“ 1 “
“ 22			22.9	“ 1 “
“ 23				“ 1 “
“ 24		89 8	46 3	“ 1 “
“ 25			46 3	“ 1 “
“ 26			46 3	Tyrode 1 “
“ 27		128 4	84 8	Serum 1 “
“ 28		191.9	90 7	“ 1 “
Mar. 1		234.5	97.5	“ 1 “
“ 2		276 4	128.5	Tyrode 1 “
“ 3		294.1	172.9	Serum 1 “

A spontaneous rejuvenation of fibroblasts after they had been cultivated in serum for 8 days and had undergone degenerative



TEXT-FIG. 1. Action of lymphocytes on fibroblasts in the presence of serum.

changes has never been observed previously. It can be attributed to the nearby presence of lymphocytes acting upon the fibroblasts. The rate of proliferation of fibroblasts has been observed to increase several fold when they grow near colonies of lymphocytes. In the above experiment, an allied phenomenon took place. As it appeared after the lymphocytes had been cultivated in serum for more than 10 days, it may be assumed that the growth-promoting principle secreted by these latter cells must have been elaborated from substances contained in the serum, and not from their own reserves.

DISCUSSION.

These experiments have brought to light the following facts: lymphocytes are able to live and multiply in a medium containing only blood serum, which does not suffice for the purpose in the case of fibroblasts. The presence, under certain conditions, of lymphocytes and large mononuclear cells in the neighborhood of a culture of fibroblasts in serum markedly increases the activity and the duration of life of the fibroblasts.

Since lymphocytes and large mononuclear cells cultivated in serum proliferate and manufacture substances which enable fibroblasts to grow, they appear to be endowed with a function of primary importance in the nutrition of the tissues. It is known that fibroblasts and epithelial cells do not synthetize protoplasm when cultivated in serum, while they obtain certain substances from embryonic tissue juice which determine an immense proliferation. As leucocytes secrete substances possessing the same growth-activating properties as embryonic tissues, it was conjectured that fibroblasts and epithelial cells, elements which cannot survive permanently on the substances contained in serum, are maintained through the action of the white cells of the blood.[4] More than 30 years ago, Renaut was lead by his anatomical studies to assume that the lymphocytes bring to the fixed cells the food material which they need.[7] Recently, Jolly[8] concluded from his investigations of the influence of starvation on the lymphoid apparatus, that lymphocytes store important chemical substances which are used by the organism more readily than are the materials from other tissues. Previous experiments[9] have shown that some of the substances secreted by leucocytes *in vivo* and *in vitro* have the same properties as embryonic tissue juice, and determine the multiplication of fibroblasts.

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[7] Renaut, J., Traité d'histologie pratique, Paris, 1893, i, pt. 2, 968.

[8] Jolly, J., *Compt. rend. Soc. biol.*, 1920, lxxxiii, 1209.

In the experiments described in this article, the source of the nitrogen used by the white cells in the manufacture of the essentials which promote the growth of the fibroblasts was investigated, and it appears that lymphocytes and large mononuclear cells find in blood serum all that is necessary for their proliferation and the elaboration of their secretions. They probably manufacture from certain compounds present in serum the food material which fibroblasts and epithelial cells require for their proliferation. Thus, they may be supposed to act as food transformers.

CONCLUSIONS.

It may be concluded that, under the conditions of the experiments:

1. Lymphocytes and large mononuclear cells can live and increase greatly in numbers in blood serum, while fibroblasts are not capable of doing so.
2. While living in serum, lymphocytes and large mononuclear cells manufacture and secrete substances which may be used as food material by the fibroblasts.
3. It is probable that lymphocytes and large mononuclear cells synthetize from the nitrogenous compounds contained in serum the substances which fibroblasts and epithelial cells require for their multiplication.

EXPLANATION OF PLATES.

PLATE 32.

FIG. 1. Living culture of lymphocytes and large mononuclears in a D-5 flask.

FIG. 2. 36 day old culture of lymphocytes in a D-5 flask.

PLATE 33.

FIG. 3. 34 day old culture of lymphocytes showing encapsulation of a foreign body.

FIG. 4. Scattered mononuclears in the peripheral part of the same culture.

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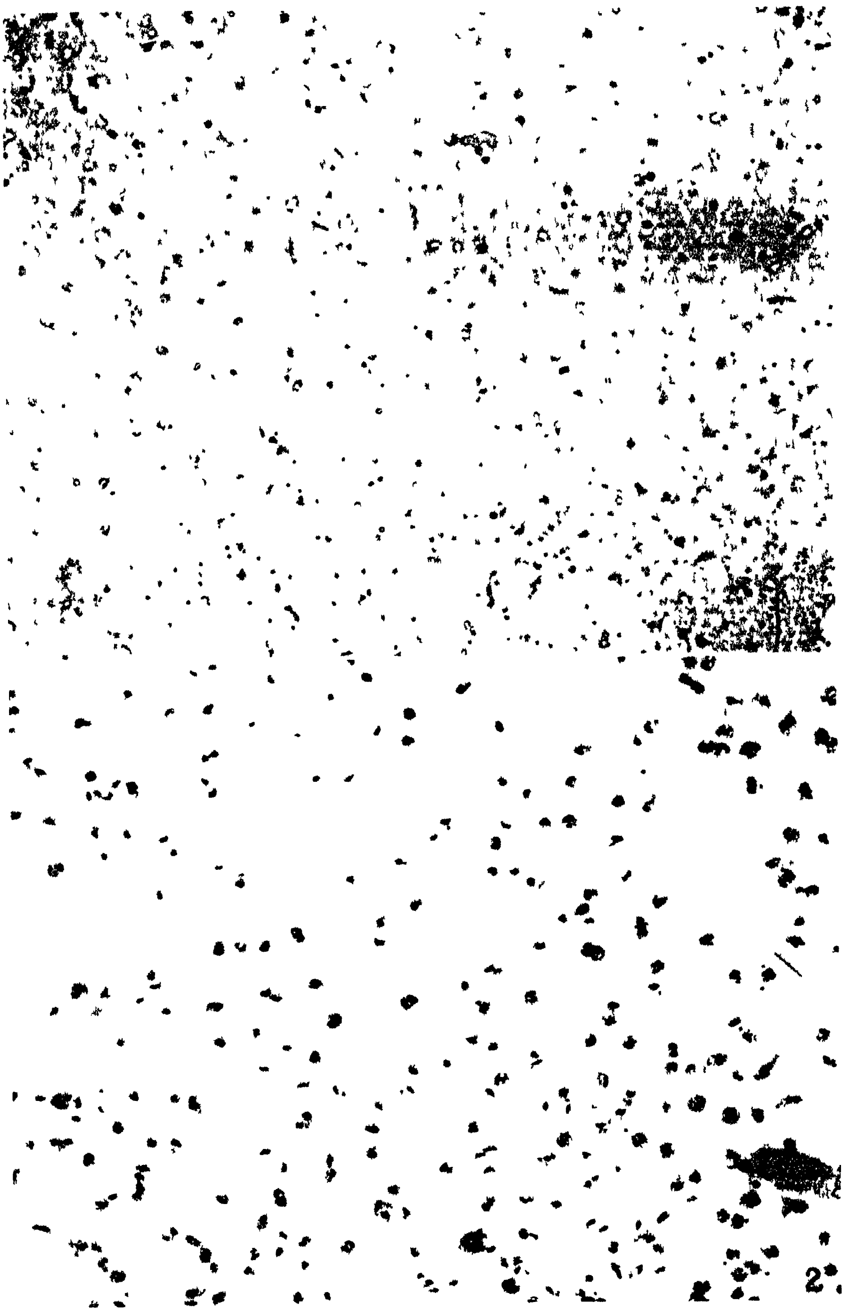
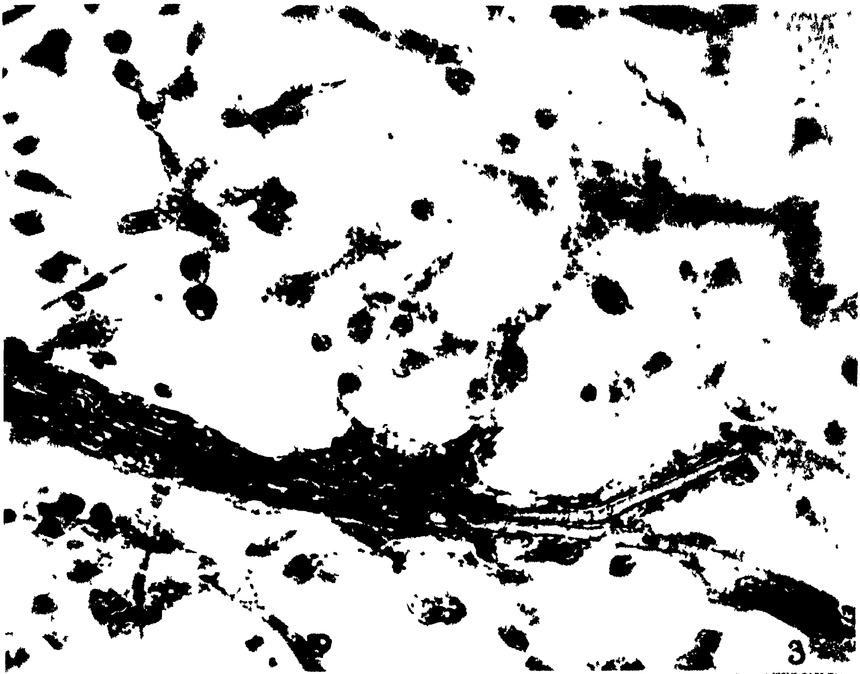
[9] Carrel, A., *J. Exp. Med.*, 1922, xxxvi, 385.



(Carrel and Ebeling Action of serum on lymphocytes)



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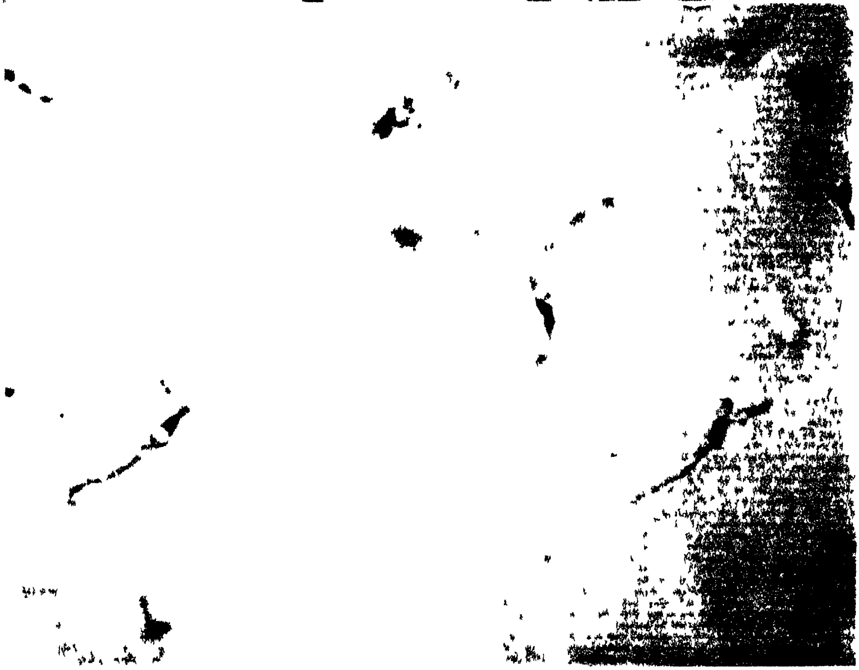

(Carrel and Ebeling: Action of serum on lymphocytes.)

[Reprinted from The Journal of Experimental Medicine, November 1, 1923, Vol. xxxviii, No. 5, pp. 521–527.]

MEASUREMENT OF THE INHERENT GROWTH ENERGY OF TISSUES.

By ALEXIS CARREL, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 5, 1923.)

It is well known that the growth energy of the organism decreases progressively with age.[1] The activity of each tissue probably declines at a different rate. Fibroblasts grow more rapidly *in vitro* from the heart of a young chick embryo than from that of an older one, and no cells migrate from a fragment of adult heart.[2] Cardiac muscle fibers lose the property of proliferating *in vitro* very early. Cultures of cutaneous epithelium can be obtained from an embryo, while no epithelial cells grow from the skin of a young chicken. Evidently, there is a definite relation between the growth energy of a given tissue and its age, which could be used for measuring the age of the organism. The rate of healing of an aseptic wound is a function of the age of the patient which is determined when the size of the wound and its index of cicatrization are known.[3] While the growth energy of the tissues slowly decreases in function of time, it may fluctuate under the influence of many other factors, as every physiological and pathological process involves a resumption or a decline of cell activity.

The variations of tissue activity *in vivo* are very probably related to those of the growth-inhibiting and promoting properties of the humors.[4] The decline of the rate of multiplication of fibroblasts cultivated in serum in function of the age of the animal from which the serum is obtained resembles the decrease of the index of cicatrization of a wound in function of the age of the patient.[5] The growth energy of the tissues decreases at the same time that the blood serum becomes more inhibiting to growth. Some years ago, it was found that fibroblasts in different conditions of activity, when cultivated in the same medium, soon grew at the same rate, and that if two halves of a culture were placed in media of different composition, they multiplied at different speeds after a short time.[6] This experiment demonstrated that the rate of cell proliferation *in vitro* depends on certain properties of the medium. We may, then, assume that the activity of a tissue *in vivo* at a given instant is probably a function of its activity at the preceding instant, and of the concentration of growth-activating and inhibiting substances in the interstitial lymph and the blood plasma.

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[1] Minot, C. S., The problem of age, growth, and death, New York, 1908.

[2] Carrel, A., *J. Exp. Med.*, 1913, xviii, 287.

[3] du Noüy, P. L., *J. Exp. Med.*, 1916, xxiv, 461.

[4] Carrel, A., *J. Exp. Med.*, 1913, xvii, 14. Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 599; 1922, xxxvi, 365. Carrel, A., *J. Exp. Med.*, 1922, xxxvi, 385. Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxvi, 645.

[5] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 599.

The knowledge of the relations between the tissues and the humors would certainly be of great importance. We can already detect the presence of the growth-activating and growth-inhibiting substances in the blood and the lymph by determining the growth index of the fluids.[6] But a method for measuring inherent cell activity remains to be developed. This inherent growth energy is not improbably proportional to the residual growth energy, that is, to the energy spent by the tissues in a non-nutritive medium until death occurs. The purpose of the experiments described in this article was to develop a technique for measuring the residual energy of fibroblasts and the relations between inherent and residual energies.

EXPERIMENTAL.

1. Measurement of the Residual Growth Energy of Fibroblasts.—A fragment of a pure strain of fibroblasts or of heart tissue was placed in a D-5 flask in 0.5 cc. of plasma and 1.5 cc. of Tyrode solution containing 5 per cent embryonic tissue juice.[7] After coagulation occurred, 1 cc. of Tyrode solution was poured on the surface of the coagulum. Every 48 hours, the fluid was aspirated and replaced by fresh Tyrode solution. Immediately after the preparation of the culture, and every 48 hours, the outline of the tissues was traced in a projectoscope, and the area measured with a planimeter. The extent of the migration of the fibroblasts and the duration of their life represented the residual activity of the tissue, which finds expression ordinarily in an S-shaped curve.

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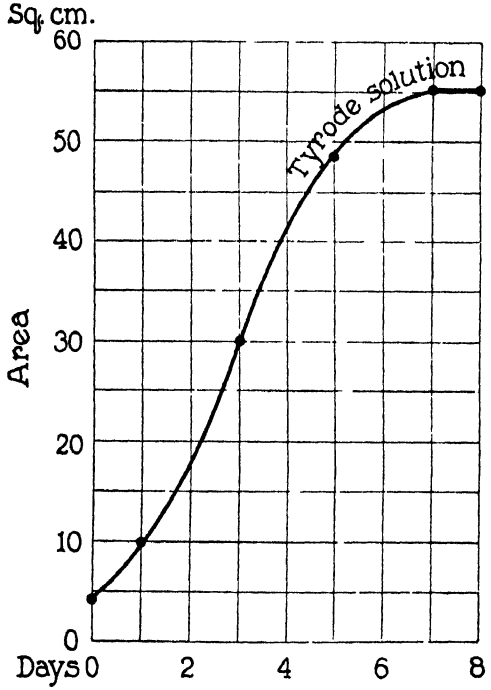
[6] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxv, 17.

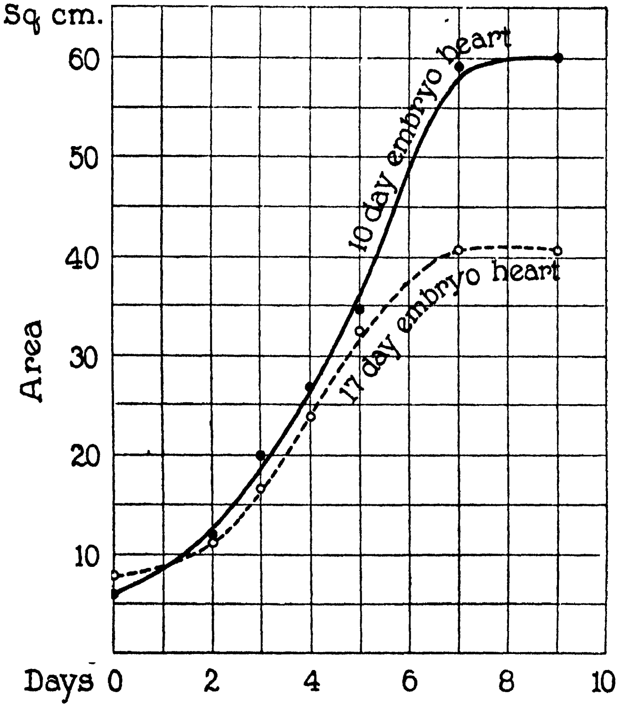
[7] Carrel, A., *J. Exp. Med.*, 1923, xxxviii, 407.

The fragments of fresh tissue usually died before the central part of the original portion disintegrated, that is, before the nutritive substances which they contained had been entirely used. The residual life of a fragment of heart of an 8 day embryo varied from 7 to 12 days. When the original fragment disintegrated early, the rate of multiplication and duration of the life of the fibroblasts were increased.



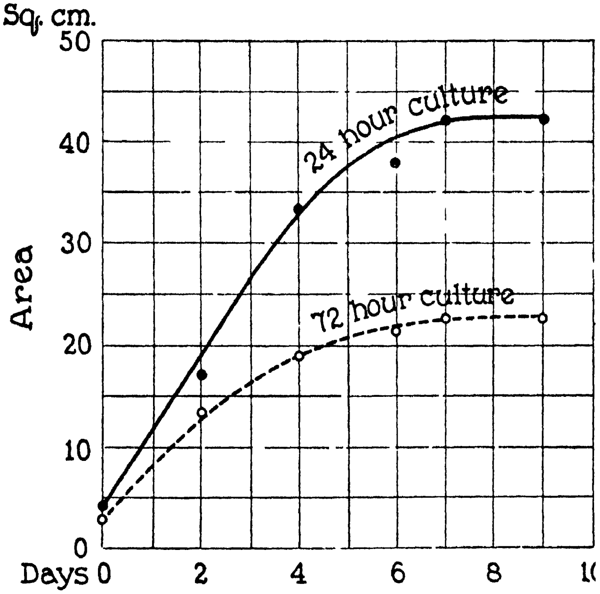
Text-Fig. 1. Residual growth energy of fibroblasts.

Fragments of a pure strain of fibroblasts used all the nutritive substances which they contained before they died. The residual life of a fragment of an 11 year old strain of fibroblasts, in normal condition, generally lasted 7 or 8 days (Text-fig. 1). Under the conditions of the experiments, the period of growth never extended beyond 9 or 10 days. During the first 2 days, there was very little difference in the rate of growth of fibroblasts cultivated in nutrient and non-nutrient media. As the solid medium was composed of plasma, and the fluid medium of Tyrode solution renewed every 2 days, the tissues were bathed in a fluid which became progressively less concentrated in serum, that is, less inhibiting. The first inflection of the curve might be due to the dilution of the medium under the influence of the Tyrode solution. From the 2nd to the 4th or 5th day, the growth was very active. The effect of starvation became apparent after 4 days. The growth slackened and the curve began the second inflection. As the solid medium was constantly in contact with Tyrode solution, the decrease in the rate of growth cannot be referred to an accumulation of catabolic substances, but to the lack of nutrient materials. It is evident that the activity of the cells in a medium composed chiefly of Tyrode solution must depend in large measure upon the preceding condition of activity.



TEXT-FIG. 2. Residual growth energy of fragments of heart of 10 and 17 day chick embryos.

2. *Residual Growth Energy of Tissues from Animals of Different Ages.*—In several experiments, two fragments of the ventricle of the hearts of 10 and 17 day chick embryos were placed in a D-5 flask, according to a technique previously described.[7] The curve expressing the residual activity, as determined by the experiment and recorded in Text-fig. 2, shows that the duration of life of both tissues was about the same, but that the growth of the older tissue was 30 per cent less extensive than that of the younger. There was certainly a marked difference in the inherent activities of the two tissues referable to the difference in age of the embryos from which they were derived.

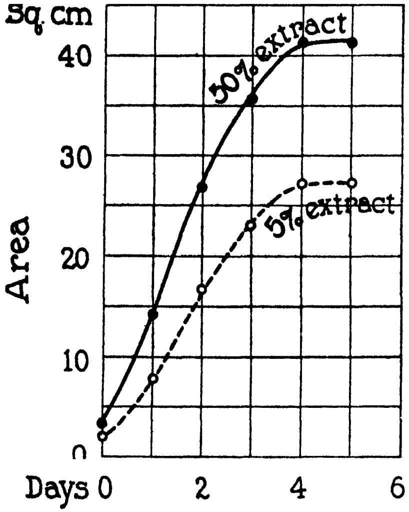


TEXT-FIG. 3. Residual growth energy of fibroblasts kept previously in a hanging drop for 24 and 72 hours respectively.

3. *Residual Growth Energy of a Pure Strain of Fibroblasts after 24 and 72 Hours Cultivation in a Hanging Drop.*—When fibroblasts are cultivated in a hanging drop of plasma and embryonic tissue juice, the S-shaped growth curve shows that great variations occur in the inherent activity of the tissues during 72 hours. The period of optimal growth lasts from about the 12th to the 48th hour. Later, the curve flattens

and becomes almost parallel to the time axis after 72 hours.[8] This second inflection is due to the spontaneous disappearance of the thermolabile growth-promoting substances of the medium and to the accumulation of catabolic products. It is probable that fragments of a pure strain of fibroblasts brought to different states of activity by cultivation for 24 and 72 hours in a hanging drop would show corresponding differences of residual activity.

Several fragments of an 11 year old strain of fibroblasts were cultivated on hollow slides in hanging drops composed of 1 volume of plasma and 1 volume of embryonic tissue juice. After 24 and 72 hours, respectively, fibroblasts were taken from the hanging drop cultures, imbedded in the medium in a D-5 flask, and their residual growth observed. The duration of life was about the same, but the extent of migration of the fibroblasts cultivated for 72 hours was about 45 per cent less than that of those cultivated for 24 hours (Text-fig. 3).



TEXT-FIG. 4. Residual growth energy of fibroblasts kept previously for 48 hours in a hanging drop containing 5 and 50 per cent embryonic tissue juice respectively.

4. Residual Growth Energy of a Pure Strain of Fibroblasts Cultivated in Media Containing 5 and 50 Per Cent Embryonic Tissue Extracts.—Two halves of a fragment from an 11 year old strain of fibroblasts were placed in media containing respectively 5 and 50 per cent embryonic tissue juice, in order to modify the inherent activity. After 48 hours, both fragments were placed in a flask in the ordinary medium and the residual activity was measured. The duration of life of both fragments was about the same, but the area covered by the cells of the fragments previously cultivated in 50 per cent embryonic tissue juice was much larger. In the experiment recorded in Text-fig. 4, the activity of the cells, as expressed by the extent of their migration, was 25 per cent greater for the tissues previously cultivated in 50 per cent embryonic tissue juice than for those cultivated in 5 per cent.

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[8] Ebeling, A. H., unpublished experiments, 1919.

CONCLUSIONS.

1. The residual growth energy of fibroblasts is expressed by the extent of their migration and multiplication in a non-nutrient medium.
2. The residual energy of fibroblasts is related to their inherent energy and the variations of the inherent energy can be ascertained by the measurement of the residual energy.

[Reprinted from THE JOURNAL OF GENERAL PHYSIOLOGY, September 20, 1923, Vol. vi,
No. 1, p. 93.]

MEMBRANE POTENTIALS AND COLLOIDAL BEHAVIOR.

REPLY TO THE NOTE BY PROFESSOR A. V. HILL.

BY DAVID I. HITCHCOCK.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 25, 1923.)

There is no real point of conflict between our views and those expressed by Professor Hill. The mere agreement of the figures for P.D., considered abstractly and apart from experimental conditions, is not a proof of the existence of a Donnan equilibrium. For example, in an experiment in which the protein were omitted altogether there would also be agreement of the P.D. values, for each would be zero; but no one would think of calling this agreement in itself a proof of the existence of a Donnan equilibrium.

The statement to which Hill has objected was not intended to be taken out of its context and used alone as a complete argument. The proof of the agreement of the P.D. values was a necessary step in testing the theory. It shows, as Hill has admitted, that the systems studied were in equilibrium. The fact that they could be in equilibrium, and still have different hydrogen or chloride ion concentrations on the opposite sides of the membrane, was by no means obvious, and required explanation. Donnan's theory furnished this explanation; and it has been pointed out that the experiments are quantitatively in accord with other deductions from the theory.

Since Donnan's theory does explain the facts, and no other theory has been proposed which can explain them in the same quantitative manner, it seems necessary to retain Loeb's original conclusion that his data have proved the applicability of Donnan's theory to these experiments with proteins.

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THE COMBINATION OF DEAMINIZED GELATIN WITH HYDROCHLORIC ACID.

BY DAVID I. HITCHCOCK.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, May 28, 1923.)

I.

INTRODUCTION.

When a protein such as gelatin is treated with nitrous acid there is an evolution of nitrogen, presumably from the reaction of the nitrous acid with NH_2 groups in the protein. This reaction is the basis of the well known Van Slyke[1] method for the quantitative determination of free amino groups. The resulting deaminized proteins, in which each NH_2 group may be assumed to have been replaced by OH, were studied by Skraup,[2] who investigated in some detail the products of their hydrolysis. Deaminized gelatin was prepared according to Skraup's method by Blasel and Matula,[3] who showed by hydrogen electrode measurements that the deaminized protein was still capable of combining with hydrochloric acid.

The present work was undertaken to compare the maximal combining capacity for hydrochloric acid of gelatin with that of deaminized gelatin. If the combination of these proteins with hydrochloric acid is true chemical combination, and if the process of deaminization consists simply in replacing NH_2 groups by OH, then it would be expected that the difference in their combining capacities should be chemically equivalent to the NH_2 groups removed. That this expectation has been confirmed is shown by the following experimental results.

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[1] Van Slyke, D. D., *J. Biol. Chem.*, 1911, ix, 185; 1912, xii, 275.

[2] Skraup, Z. H., *Monatsh. Chem.*, 1906, xxvii, 653; 1907, xxviii, 447.

[3] Blasel, L., and Matula, J., *Biochem. Z.*, 1913–14, lviii, 417.

II.

The Deaminizing Reaction.

It was found by Van Slyke[1] that the deaminizing reaction took place rapidly and quantitatively if the amino substance was treated with a great excess of a solution containing equivalent amounts of sodium nitrite and acetic acid. The preparation of Skraup was obtained under similar conditions, but after allowing the mixture to stand overnight he heated the acid solution for 2 hours on a water bath and then precipitated the deaminized gelatin repeatedly by saturation with ammonium sulfate.

In order to determine whether this treatment caused any further reaction than that occurring in the Van Slyke apparatus, measurements were made of the amino nitrogen in gelatin, by Van Slyke's[1] method, and of the total nitrogen in gelatin and in deaminized gelatin, by the Kjeldahl method.[4] Three lots of deaminized gelatin were used. No. 1 was prepared from Cooper's commercial gelatin according to Skraup, except that it was precipitated only once by ammonium sulfate, while No. 2 was part of the same lot which was not heated on the water bath and not precipitated, but was dialyzed under pressure in collodion cylinders after standing overnight in the nitrous acid solution. The dialysis was started in running tap water; after about 4 days the contents of the bags were brought to pH 3.6 with hydrochloric acid, and then dialyzed for a week against repeated changes of hydrochloric acid of pH 3.6; finally the bags were kept for 3 days in repeated changes of distilled water. This reduced the specific conductivity of the resulting 3 per cent solutions to less than 10^{-4} reciprocal ohms. The concentrations of these solutions were determined by drying 25 cc. samples to constant weight at 110°C., and 25 cc. samples were likewise used for the Kjeldahl determinations. The gelatin analyzed was a solution of isoelectric gelatin which had been prepared from Cooper's gelatin as described by Loeb.[5] Preparation 3 of deaminized gelatin was made by dialyzing the residues from the Van Slyke determinations, for which the initial material was isoelectric gelatin.

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[4] Sherman, H. C., Methods of organic analysis. New York, 2nd edition, 1912, 291.

[5] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, 35.

By the Van Slyke method the following figures were found for the percentage of amino nitrogen, using samples containing from 0.8 to 1.2 gm. of dry gelatin: 0.525, 0.505, 0.582, 0.662, 0.540, 0.514; average, 0.555 per cent. This agrees with the results of Van Slyke and Birchard,[6] who found 3.12 per cent of the total nitrogen in gelatin to be amino nitrogen; taking the total nitrogen as 17.96 per cent (see below), their figure for amino nitrogen becomes 0.560 per cent of the gelatin. Similar figures were obtained by Northrop,[7] who found the normality of 1 per cent gelatin to be 0.0036 or 0.0038 with respect to NH_2 groups; accordingly his value for the percentage of amino nitrogen in dry gelatin is 10 × 0.0037 × 14.01 = 0.519 per cent.

By the Kjeldahl method the following figures for total nitrogen were obtained.

Gelatin....	18.02	17.94	17.97	17.89	average, 17.96 per cent.
Deaminized gelatin No. 1. .	16.98	16.94	16.99		“ 16.97 “ “
“ “ “ 2....	17.46	17.35	17.41	17.37	“ 17.40 “ “

It is evident that the difference between the figure for gelatin and that for deaminized gelatin No. 2 is almost exactly equal to the percentage of amino nitrogen removed in the Van Slyke analysis. In the case of deaminized gelatin No. 1, which was prepared according to Skraup, with heating, the loss of nitrogen is considerably greater, indicating that a more extensive reaction has taken place than the simple removal of free amino groups. This was corroborated by the fact that in the dialysis of No. 1 much more material was lost by diffusion through the collodion than was the case with No. 2.

These figures may be summarized as follows:

1 gm. gelatin = 0.00555 gm. or 0.000396 equivalents amino N.
1 “ “ = 0.1796 “ “ 0.01282 “ total “
1 “ deaminized gelatin No. 2 = 0.1740 “ “ 0.01242 “ “ “
Loss in deaminization = 0.0056 “ “ 0.00040 “ N.

Equal weights of gelatin and deaminized gelatin are taken as chemically equivalent, because the formula weight of the group OH, 17, is so close to that of the NH_2 group, 16, and because the groups affected are such a small part of the weight of the protein. These results indicate that if the preparation of deaminized gelatin is carried out without heating, the reaction is simply a replacement of NH_2 by OH, as in the case of ordinary aliphatic amines.

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[6] Van Slyke, D. D., and Birchard, F. J., *J. Biol. Chem.*, 1913–14, xvi, 539.

[7] Northrop, J. H., *J. Gen. Physiol.*, 1920–21, iii, 715.

This simple conception of the reaction occurring in the preparation of deaminized gelatin was confirmed by analyzing the product by the Van Slyke method; the percentage of amino nitrogen found was 0.025 for Preparation 1 and 0.029 for Preparation 2. It is believed that these figures should both be zero, since the volumes of nitrogen obtained, after correction for the blank from the reagents, were only 0.16 and 0.15 cc. The absence of amino nitrogen in several other preparations of deaminized gelatin, prepared both with and without heating, was further confirmed by the formol titration method of Sörensen.[8] In no case was there any difference between the titrations with and without formaldehyde, if proper correction was made for the acidity of the formaldehyde solution itself. It may be stated that this is quite at variance with the results reported by Herzig and Lieb,[9] who found over twice as much amino nitrogen in gelatin as any of the authors quoted above, and found similar high figures for amino nitrogen in deaminized gelatin, even after it had been twice deaminized.

III.

Combination of Deaminized Gelatin with HCl.

In order to calculate the combining capacity of deaminized gelatin for acid, it was necessary to know the isoelectric point of the material. In the work of Blasel and Matula[3] this was not considered, and their curve therefore does not represent the true amounts of combined acid.

The isoelectric point of a protein has been shown by Loeb[10] to coincide with a minimum of osmotic pressure. Accordingly measurements were made of the osmotic pressure at 24°C. and of the pH of the protein solutions at equilibrium, in the manner described by Loeb. The results obtained with 1 per cent solutions of Preparation 1, and other preparations which had been heated even more than 2 hours on

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[8] Sörensen, S. P. L., *Compt. rend. trav. Lab. Carlsberg*, 1907, vii, 1; *Biochem. Z.*, 1908, vii, 64.

[9] Herzig, J., and Lieb, H., *Z. physiol. Chem.*, 1921, cxvii, 1.

[10] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, 37.

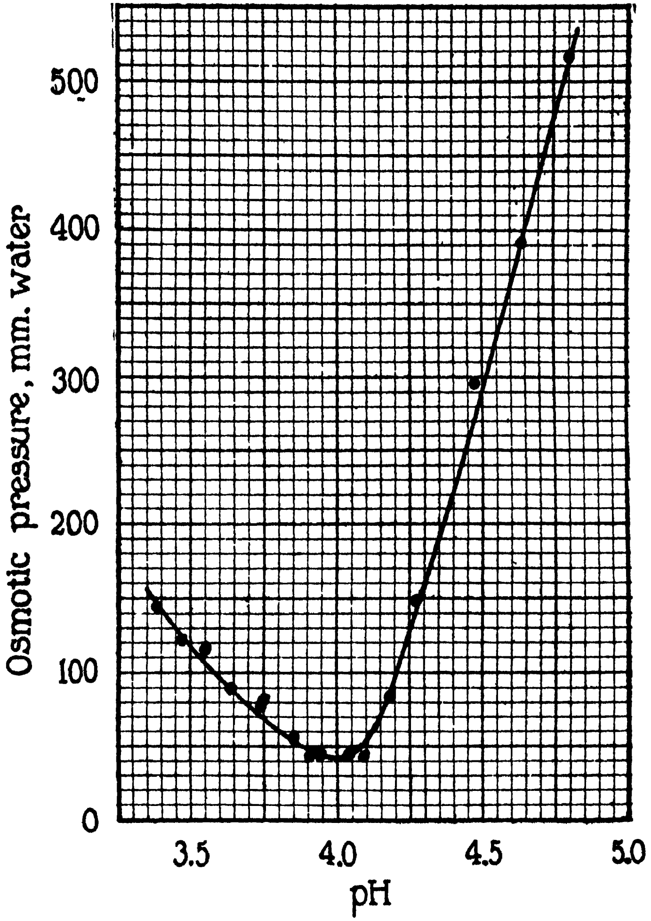

Fig. 1. Effect of pH on osmotic pressure of 1 per cent deaminized gelatin.

the water bath, showed a minimum of osmotic pressure at pH 3.5 to 3.8. Preparation 2, however, exhibited a minimum of osmotic pressure at pH 4.0, as is shown by Fig. 1.

This location of the isoelectric point was confirmed in another way in the case of Preparation 3, which was also prepared without heating. The protein was coated on particles of collodion by mixing 50 cc. of a

0.1 per cent solution with 50 cc. of a suspension of collodion particles, prepared as described by Loeb,[11] and allowing the mixture to stand overnight. The mixture was then centrifuged, and the sediment was again put into suspension by trituration in a mortar with about 15 cc. of distilled water. A series of acetate buffers was prepared as described by Michaelis,[12] 0.01 M with respect to sodium acetate, and 5 drops of the concentrated suspension were added to 25 cc. of each buffer. Measurements were made of the velocity of migration of the particles in an electric field, using the microscopic apparatus of Northrop.[13] For these measurements the writer is indebted to Mr. M. Kunitz, who found that the direction of migration changed its sign between pH 3.7 and 4.0. 10 cc. samples of the various suspensions were allowed to stand overnight in test-tubes, and it was found that the suspension at pH 3.9 settled clear, those differing by about pH 0.1 or more being cloudy. In this work the pH determinations were made with the hydrogen electrode at 33°C. in Clark cells with a saturated KCl junction, and were based on a pH value of 1.037 for 0.1000 M HCl.

The determination of the combining capacity of deaminized gelatin was carried out by a method similar to that already employed in the case of gelatin.[14,15] This consists in measuring the pH in protein solutions containing various amounts of acid, and subtracting from the total acid concentration the amount of acid required to give the same pH to an equal volume of water. The difference represents the amount of acid combined with the protein, and by dividing by the protein concentration figures may be obtained for the amounts of acid combined with 1 gm. of protein at different pH values. It was found in the case of gelatin[15] that the amounts so obtained for a given pH were independent of the protein concentration, and that between pH 1 and 2 the amount of hydrochloric acid combined with 1 gm. of gelatin was constant.

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[11] Loeb, J., *J. Gen. Physiol.*, 1922–23, v, 109.

[12] Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914, 184.

[13] Northrop, J. H., *J. Gen. Physiol.*, 1921–22, iv, 629.

[14] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, 51.

[15] Hitchcock, D. I., *J. Gen. Physiol.*, 1921–22, iv, 733.

In the case of deaminized gelatin the method was modified by performing the titration with a single sample of protein, instead of making up a fresh sample to the same volume for each pH determination. A vessel of the type described by Bovie[16] was used, with an electrode of platinized platinum wire and bubbling hydrogen. Contact was made with a saturated potassium chloride bridge through a tube not quite tightly closed by a ground glass stopper. Junctions of this type were tested by Lamb and Larson[17] and recommended by LaMer and Baker.[18] The salt bridge was connected to a saturated potassium chloride calomel cell, and the whole apparatus was kept in a water bath at 33° ± 0.1. 50 cc. of the protein solution were placed in the cell, and after the electrode had come to equilibrium the titrations were made by running in standard HCl solution from a burette. After each addition of acid, readings of the E.M.F. were taken at intervals until they became constant for 2 minutes. The E.M.F. measurements were made with a Leeds and Northrup portable potentiometer reading to 0.5 millivolt.

The calculation of the combined hydrochloric acid was performed as follows:

Let a = No. of cc. of HCl added to e cc. of protein solution.
b = concentration of HCl in burette, mols per liter.
c = initial concentration of protein solution, gm. per liter.
d = concentration of HCl having the same pH in water alone, mols per liter.
e = initial volume of protein solution, cc.

Then $a + e$ = volume of mixture, cc.
$\frac{ab}{a+e}$ = concentration of HCl in mixture, mols per liter.
$\frac{ab}{a+e} - d = \frac{ab - ad - ed}{a+e}$ = combined HCl, mols per liter.
$\frac{ec}{a+e}$ concentration of protein in mixture, gm per liter.
$\frac{ab - cd - ed}{ec}$ = mols of HCl combined with 1 gm. of protein.

The values of d were obtained from the curve constructed in connection with the previous work on gelatin;[15] this curve was checked by titrating 50 cc. of water with 0.201 M HCl in the new apparatus. In case the original protein solution is not at the isoelectric point, the values for the combined acid must be corrected by adding or subtracting a constant amount to make the point of zero combination coincide with the isoelectric point. The only assumptions involved in the calculation are those used previously; namely, that the same concentration of uncombined HCl is required to give the same pH to equal volumes of water and of protein chloride solution, and that there is no acid combined with the protein at its isoelectric point.

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[16] Bovie, W. T., *J. Am. Chem. Soc.*, 1922, xliv, 2892.
[17] Lamb, A. B., and Larson, A. T., *J. Am. Chem. Soc.*, 1920, xlii, 229.
[18] LaMer, V. K., and Baker, L. E., *J. Am. Chem. Soc.*, 1922, xliv, 1954.

TABLE I.

Titration of Deaminized Gelatin with Hydrochloric Acid.

A		B		C		D	
HCl	pH	HCl	pH	HCl	pH	HCl	pH
cc.		*cc.*		*cc.*		*cc.*	
0	3.88	0	4.02	0	4.02	1.20	3.61
0.50	3.18	0.94	3.66	1.29	3.53	1.90	3.35
0.99	2.78	2.81	2.94	2.94	2.88	2.80	2.99
1.99	2.29	4.58	2.32	4.92	2.21	4.40	2.45
3.17	2.03	6.58	2.00	5.93	2.02	7.10	1.98
4.51	1.85			6.93	1.89		
				8.43	1.75		

A. Preparation 3. Initial volume, e = 50 cc. Concentration of protein, c = 6.99 gm. per liter. Concentration of HCl, d = 0.201 M.

B and C. Preparation 2. Initial volume, e = 50 cc. Concentration of protein, c = 29.69 gm. per liter. Concentration of HCl, d = 0.201 M.

D. Preparation 2. In this case the solutions were made up to constant volume before measuring pH. The figures are cc. of 0.1003 M HCl contained in 35 cc. of solution containing also 0.743 gm. of deaminized gelatin.

The results of the titration experiments are given in Table I. From these results the amounts of hydrochloric acid combined with 1 gm. of protein were calculated in the way just described; the quantities so obtained are plotted in Fig. 2. The correction mentioned was necessary only in the case of Experiment A; in the other cases the solution happened to be at the isoelectric point when the dialysis was stopped. The curve indicates that deaminized gelatin has a maximum combining capacity of 0.00044 mols of HCl per gm. of protein.

IV.

CONCLUSIONS.

In a previous paper[15] it was stated that 1 gm. of gelatin could combine with 0.00092 mols of HCl. This figure should be corrected by subtracting the amount of acid necessary to shift the zero point from pH 4.78 to 4.70, the isoelectric point of gelatin. This correction makes the value for the maximum combined HCl 0.00089 mols per gm. of gelatin, and changes the combining weight of gelatin to about 1,120.

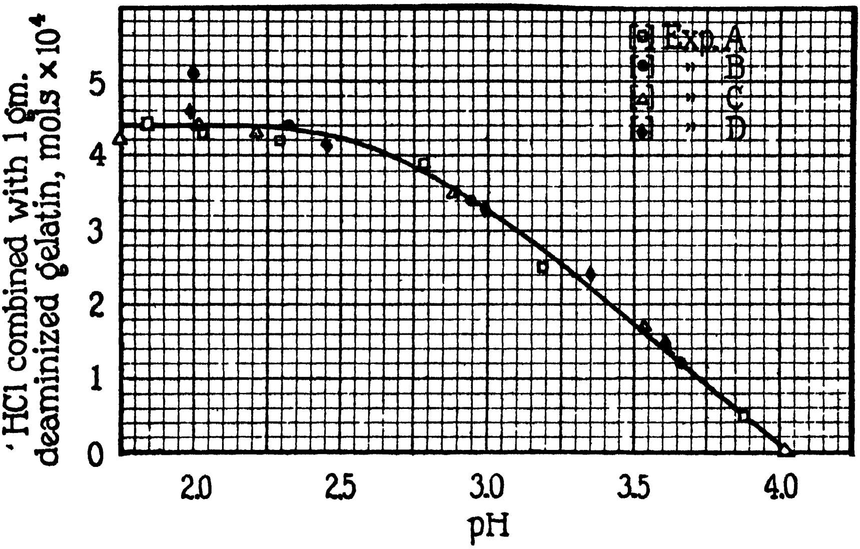

FIG. 2. Combination of deaminized gelatin with HCl.

The number of equivalents of amino nitrogen removed in deaminizing 1 gm. of gelatin was found to be 0.00040, and this was checked by the loss in total nitrogen. The difference between the maximum combining capacities for HCl of 1 gm. of gelatin and 1 gm. of deaminized gelatin is 0.00089 − 0.00044 = 0.00045. Considering the limitations of the methods which had to be employed to get these figures, the agreement is good.

This means, then, that the loss in combining capacity for HCl suffered by 1 gm. of gelatin in being deaminized is chemically equivalent to the number of amino groups removed. The combining capacity for HCl still retained by deaminized gelatin is presumably to be ascribed to the NH groups which are not attacked by HNO_2. For each atom of nitrogen lost in the deaminizing reaction, the protein loses the capacity to combine with one hydrogen ion. Therefore the present work constitutes a new indication of the truly chemical nature of the combination between protein and acid.

V.

SUMMARY.

1. The analysis of isoelectric gelatin by the Van Slyke method indicates 0.00040 equivalents of amino N per gm. gelatin.
2. If deaminized gelatin is prepared without heating, the product contains less nitrogen than the original gelatin by an amount equal to 0.00040 equivalents N per gm. protein.
3. Deaminized gelatin, prepared either with or without heating, contains no amino nitrogen detectable with certainty by either the Van Slyke or the formol titration method.
4. The isoelectric point of deaminized gelatin prepared without heating is at pH 4.0.
5. The maximum combining capacity of this protein for HCl is 0.00044 equivalents per gm.
6. The maximum combining capacity of gelatin for HCl should be corrected to 0.00089 equivalents per gm.
7. The difference between these maximum combining capacities, 0.00045, is nearly equivalent to the loss in amino or total nitrogen occurring in the deaminizing reaction.
8. This equivalence constitutes a new indication that the combination of protein with acid is chemical combination.

The writer is indebted to Dr. Jacques Loeb for suggesting this work.

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ON THE LOCATION OF THE FORCES WHICH DETERMINE THE ELECTRICAL DOUBLE LAYER BETWEEN COLLODION PARTICLES AND WATER.

BY JACQUES LOEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, July 12, 1923.)

I.

The electrical double layer surrounding solid particles suspended in water is assumed to be due to a difference in the concentration of the oppositely charged ions of an electrolyte in the enveloping film (*i.e.* the film of water which adheres to and moves with the particle in an electrical field) and the opposite film. This difference in concentration is generally ascribed to a preferential adsorption of one kind of a pair of oppositely charged ions by the suspended particle. Such a definition is inadequate inasmuch as the term adsorption includes two entirely different kinds of forces; namely, first, forces inherent exclusively in the water, and second, forces of attraction between the suspended particle and the adsorbed ions. Forces inherent exclusively in the water are responsible for the cataphoretic migration of gas bubbles in water in which the gas bubbles are negatively charged since the nature of the gas is of no influence on the P.D. McTaggart[1] suggested that in this case there exists an excess of OH ions in the film of water which envelops the gas bubble (adhering to and moving with it); while the opposite film has an equal excess of H ions. The writer has recently suggested that this electrical double layer might be due to an orientation of the molecules of water at the surface of the water whereby the oxygen atoms of the water molecules are turned outwards and the two hydrogen atoms or ions are turned inside into the water.[2] Such an orientation of the molecules of water at the free surface might also explain the well known fact that the smallest particles torn off mechanically from the surface of water are negatively charged, since it would be likely that if fine particles are torn off from the surface they would carry an excess of OH ions with them, leaving behind an excess of H ions. It is, however, possible that the anion responsible for the negative charge in these cases might not have been the OH ion but the anion of an electrolyte present in small traces. Our recent experiments have led us to suspect that such impurities play a greater rôle in the cataphoretic P.D. of solid particles in "pure water" than has been realized. As has been pointed out by Lenard[3] and McTaggart, there can be no doubt that the electrical double layer is in these cases determined entirely by forces inherent in the water itself. There is no reason why these forces should not also influence the cataphoretic P.D. at the boundary of solid particles and water.

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[1] McTaggart, H. A., *Phil. Mag.*, 1914, xxvii, 297; xxviii, 367; 1922, xliv, 386.

[2] Loeb, J., *J. Gen. Physiol.*, 1922–23, v, 513.

The second source of an unequal distribution of a pair of oppositely charged ions at the boundary of suspended particles and water are forces inherent in the suspended particles whereby ions of a definite sign, either positive or negative, are attracted by the particles. We have a clear case of the action of such forces when a substance dissolved in the water, *e.g.* a protein, forms a durable irreversible film on the surface of the particle.

II.

The influence of ordinary electrolytes, such as HCl, H_2SO_4, NaOH, NaCl, $CaCl_2$, $LaCl_3$, Na_2SO_4, and $Na_4Fe(CN)_6$, on the cataphoretic P.D. of collodion particles in water has already been described,[4] but for the convenience of the reader Figs. 1 and 2 are reproduced from a preceding paper, illustrating the effect of the electrolytes mentioned on the cataphoretic P.D. of collodion particles.

Fig. 1 shows the effect of different concentrations of HCl, H_2SO_4, and NaOH on the cataphoretic P.D. of collodion particles measured by microscopic observation of the rate of migration of the particles as described in a previous paper,[4] and calculated from these measurements with the Helmholtz-Perrin formula (multiplying by 14 the velocity of migration in cm.$^{-4}$ for a gradient of 1 volt per cm. at 20°C. The distilled water, before acid or alkali was added, had a pH of about 5.8 and the cataphoretic P.D. of the particles was about 30 millivolts,

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[3] Lenard, P., *Ann. Physik*, 1915, xlvii, 463.

[4] Loeb, J., *J. Gen. Physiol.*, 1922–23, v, 109.

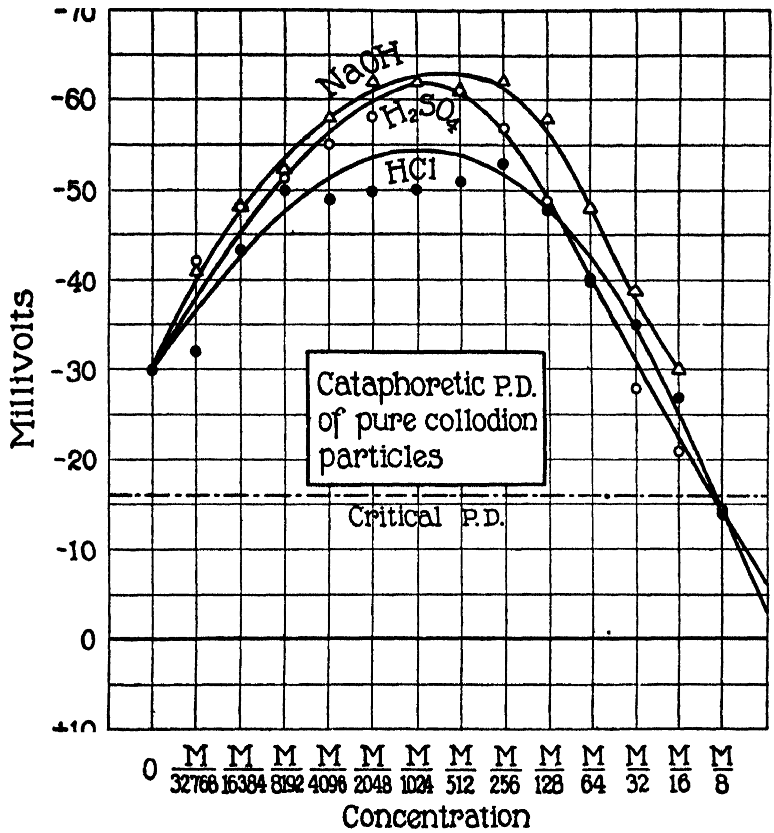

FIG. 1. Influence of acid and alkali on the cataphoretic P.D. between collodion particles and water. The original pH of water was about 5.0. The abscissæ are the concentrations of acids or alkali, the ordinates are the P.D. in millivolts. The collodion particles were negatively charged. The line marked "Critical P.D." (at 16 millivolts) is the P.D. below which the collodion suspension is no longer stable.

the particles being negatively charged. The addition of either acid or alkali increased this negative charge until a maximum was reached which was slightly higher for NaOH than for HCl, being about 65 millivolts in the case of NaOH and about 55 millivolts in the case of HCl. This maximum was reached when the concentration of acid or alkali was between M/1,000 and M/500. A further increase in concentration of the acid or alkali depressed the P.D. again.

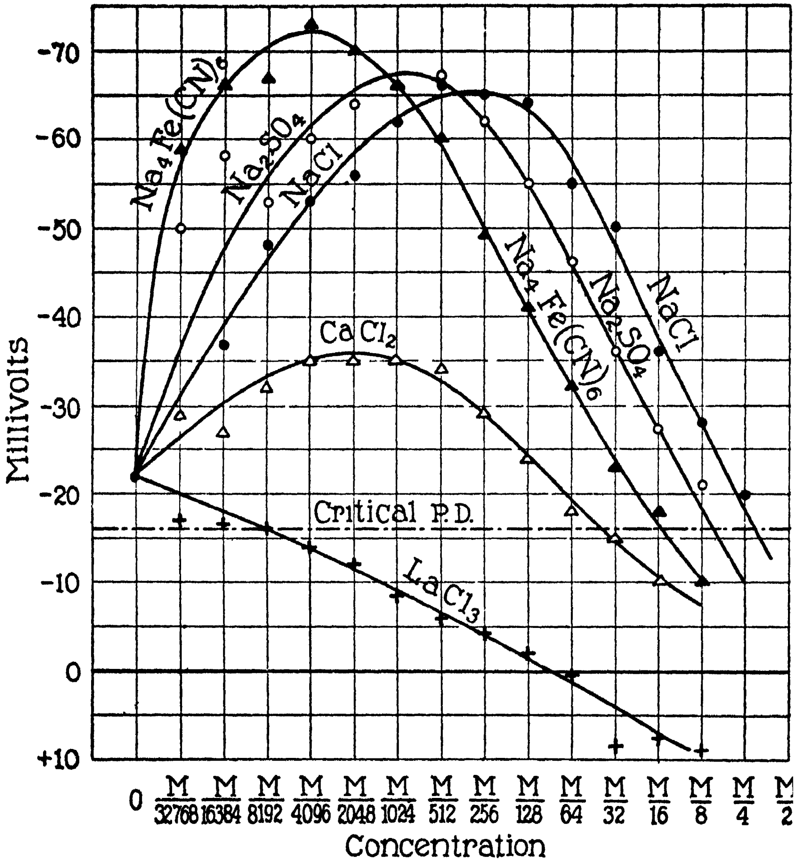

FIG. 2. Influence of $Na_4Fe(CN)_6$, Na_2SO_4, NaCl, $CaCl_2$, and $LaCl_3$ on the P.D. at pH 5.8. Addition of little salt with monovalent cation raises the P.D. to about 70 millivolts and the more rapidly the higher the valency of the anion. With $CaCl_2$ only a slight rise and with $LaCl_3$ no rise occurs in the concentrations used. In concentrations above M/64 $LaCl_3$ causes a reversal of the sign of charge of the particles.

What interests us here are the following two facts: that the collodion particles are always negatively charged and that the addition of acid or alkali at first increases the P.D. and the more so the higher the concentration of the acid or alkali added, as long as the concentration remains below a concentration somewhere between M/1,000 and M/500. This means that no matter whether we add acid or alkali to water of pH 5.8, the enveloping film of water (adhering to and moving with the collodion particle) will have an excess of anions. The fact that the addition of acid to distilled water renders the collodion particles more negative is very striking and it suggests that collodion might behave like a positively charged body always combining with anions even in acid solution.

Fig. 2 gives the effect of different concentrations of NaCl, Na_2SO_4, $Na_4Fe(CN)_6$, $CaCl_2$, and $LaCl_3$ on the cataphoretic P.D. of collodion particles in water at pH of about 5.8. Without salt the P.D. of the particles was about 22 millivolts, but rose at first with the addition of salt, and the more rapidly the higher the valency of the anion, until a maximum of about 70 millivolts was reached, after which a further increase in the concentration of the salt caused a diminution of the P.D. The rise was considerably less when a salt like $CaCl_2$ (or $MgCl_2$, $BaCl_2$) was used, and apparently no rise occurred when $LaCl_3$ was added. In this case a depression of the P.D. occurred even when the concentration of the salt was as low as M/32,000. $LaCl_3$ reverses the sign of charge of the double layer at a concentration of between M/128 and M/64. None of the salts with monovalent or divalent cation causes such a reversal in concentrations at which the P.D. can still be conveniently measured. The fact that trivalent or tetravalent ions may reverse the sign of cataphoretic charge is a well known and universal phenomenon. McTaggart found that $LaCl_3$ and $Th(NO_3)_4$ even make gas bubbles positive.[1]

The fact which interests us in Fig. 2 is that salts with monovalent and divalent cations at first raise the negative charge of the particles in cataphoresis (that is, as long as the concentration of the salts does not exceed a certain value). It may be mentioned incidentally that LiCl and KCl act on the cataphoretic P.D. almost quantitatively like NaCl. Since in all these cases the enveloping film has an excess of anions which at first increases with the concentration of the electrolytes ($LaCl_3$ or $ThCl_4$ excepted), it again looks as if the excess of anions in the film might be due to a preferential attraction of anions by the collodion particle. With the aid of dyes and proteins it can be shown, however, that cations of these latter substances can form durable films on collodion, but that the anions cannot do so. When collodion particles are put into a solution of an acid or basic dye, it is found that at pH 5.8 they remove the dye from the solution and are themselves stained correspondingly when the dye is basic but less or not at all when it is acid. They thus "adsorb" dye cations, *e.g.* basic fuchsin, neutral red, malachite green, but not dye anions, *e.g.* acid fuchsin, methyl orange, or congo red. When solutions of gelatin or albumin are used, the collodion particles are covered with a film of gelatin or of crystalline egg albumin when the protein is a cation (*e.g.* in the case of albumin chloride), but not when the protein is an anion (*e.g.* in the case of Na albuminate). This suggests that the increase in the negative charge of the collodion particles caused by the addition of ordinary crystalloidal electrolytes, as expressed in Figs. 1 and 2, cannot with any degree of certainty be attributed to the chemical forces inherent in the collodion, but must be attributed to forces inherent in the water, unless further evidence to the contrary is furnished.

III.

Solutions of six dyes were prepared in water of pH 5.8 in concentrations varying from M/2,000,000 to about M/256, *i.e.* those concentrations in which it was possible to measure the rate of motion of the particles in the solution of the dye with the aid of dark field illumination.

Fig. 3 gives the results. A comparison of Figs. 2 and 3 suggests that the salts of the three acid dyes, congo red, methyl orange, and acid fuchsin, act like NaCl or $Na_4Fe(CN)_6$; namely, the addition of the dye raises at first the cataphoretic P.D. until a maximum is reached which lies at a molecular concentration of the dye between M/4,096 and M/1,000; after which a further increase in the concentration of the dye causes a diminution of the P.D. The ions of the dye are in this case anions which cannot form a film on the surface of the collodion particles.

The influence of the three basic dyes on the cataphoretic P.D. is entirely different. These dyes do not raise the cataphoretic P.D. of the collodion particles but depress it so powerfully that in concentra-

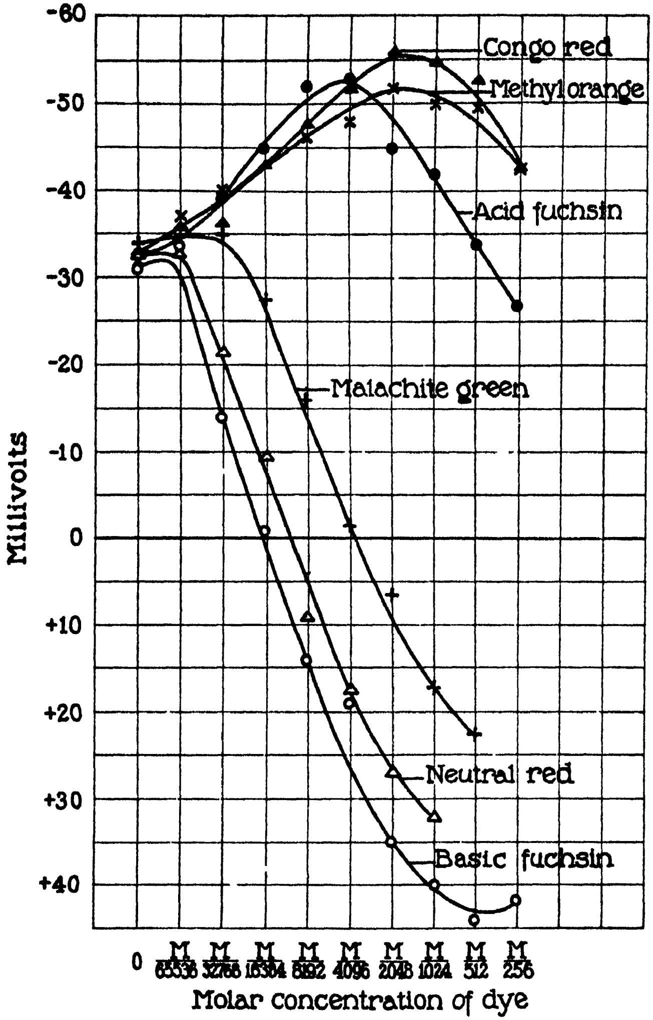

FIG. 3. Influence of dye salts on the cataphoretic P.D. of collodion particles originally of pH 5.8. Basic dyes (basic fuchsin, neutral red, and malachite green) depress the P.D. and reverse its sign at low concentrations; acid dyes (acid fuchsin, methyl orange, and congo red) act in the way of ordinary salts like NaCl (see Fig. 2).

tions between M/16,000 and M/4,000 the sign of the charge of the particles is already reversed, the enveloping film of water assuming a positive charge. In the case of basic fuchsin the P.D. becomes 40 millivolts at a concentration of about M/1,000 or M/500, the particles being positively charged. In this case the dye exists as cations and

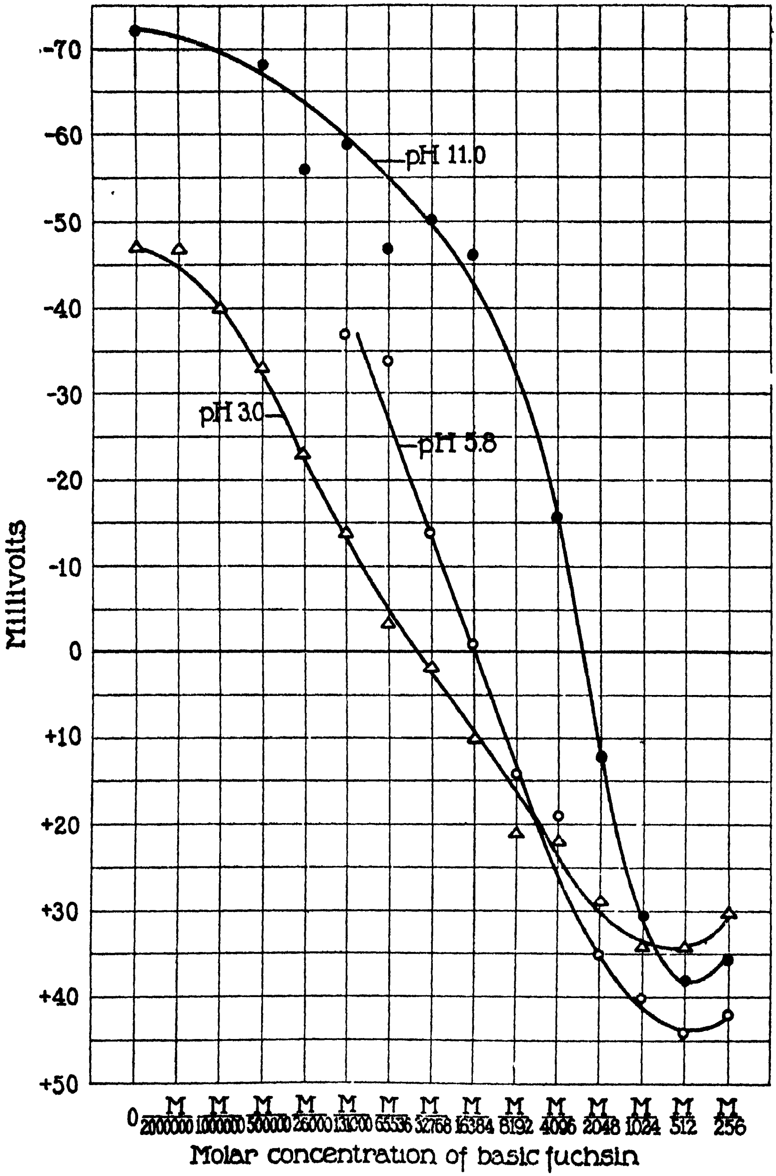

FIG. 4. A basic dye (basic fuchsin) depresses the P.D. of collodion particles and reverses its sign not only at pH 5.8 but also at 3.0 and 11.0.

these cations form a visible durable film on the surface of the collodion partcles. The migration of the particles was measured immediately afterthe collodion particles were put into these dyes. The depressing and reversing action of the basic dyes, *e.g.* basic fuchsin, occurs not only when the basic dye is dissolved in ordinary distilled water at pH 5.8 but also when it is dissolved in N/1,000 HCl or N/1,000 NaOH (*e.g.* at pH about 3.0 and 11.0) as shown in Fig. 4. The concentration at which the dye reverses the sign of charge of the particles increases with the pH.

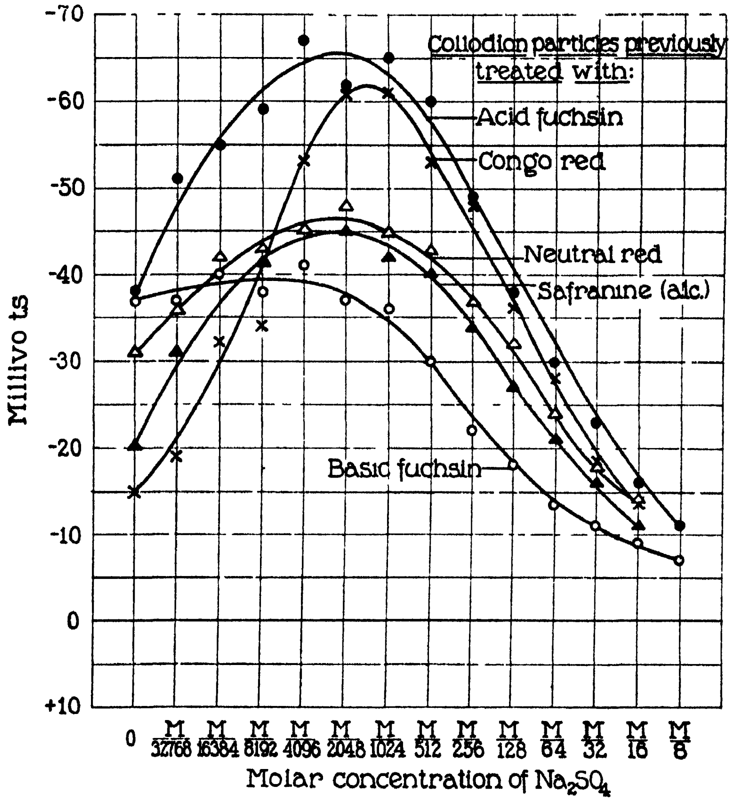

FIG. 5. Proof that the film formed on collodion particles by a previous treatment with dye cations (basic fuchsin, neutral red, safranine) lowers the effect of Na_2SO_4 on the P.D., while the effect of this salt on the P.D. of collodion particles previously treated with dye anions (acid fuchsin, congo red) is the same as upon non-treated particles. In acid dyes no film of dye is formed on the particles.

The basic dyes form a more or less durable film on the surface of the collodion particles and it seemed of interest to study to what extent such a film might alter the influence of ordinary salts on the cataphoretic P.D. of the particles. Collodion particles were kept for 24 hours in 1/10 per cent solution of different dyes. They were then freed from the dye with the aid of the centrifuge and suspended in water of pH 5.8 (free from dye). Those which had been kept in basic dye were stained, while those that had been kept in the acid dye were not stained. It was expected that the latter particles would behave like ordinary particles of collodion not previously treated with any dye; while the particles kept over night in a solution of a basic dye would retain at their surface a number of dye cations which would diminish the negative charge imposed on the particle by the anions of any ordinary salt like Na_2SO_4. After having been washed in water of pH 5.8, the collodion particles were put into solutions of Na_2SO_4 of different concentrations and their cataphoretic P.D. was measured at pH 5.8 in these solutions. Fig. 5 gives the results. The collodion particles previously treated with acid dyes (acid fuchsin or congo red) behaved like non-treated collodion particles, assuming a maximal P.D. of over 60 millivolts. The particles previously treated with basic dyes and which were slightly stained assumed a maximal P.D. of only 45 millivolts or less. The ions of the dye left at the surface of the collodion particles therefore diminished the negative charge due to the excess of SO_4 ions forced into the stratum of water touching the collodion particles. The enveloping film of the collodion particle may then in this case be conceived as being a mosaic of dye cations (held chemically at the surface of the collodion particles) and of SO_4 and possibly OH ions driven into the enveloping film by forces inherent in the water.

The experiment was repeated with $CaCl_2$ as the testing salt and the same difference was found. The P.D. of the collodion particles previously treated with basic fuchsin was depressed more by $CaCl_2$ than the P.D. of the particles previously treated with acid fuchsin (Fig. 6).

It is, however, worthy of notice that the enveloping film did not assume a positive charge at pH 5.8 when the collodion particles had previously been stained with a basic dye; and furthermore, it is worthy of notice that in Fig. 5 the character of the curves expressing the influence of Na_2SO_4 on the cataphoretic P.D. is the same in the stained and the non-stained particles of collodion. The only difference is a slight diminution of the P.D. in the stained particles. One might be tempted to assume that the dye cations formed only a small part of the area of the enveloping film.

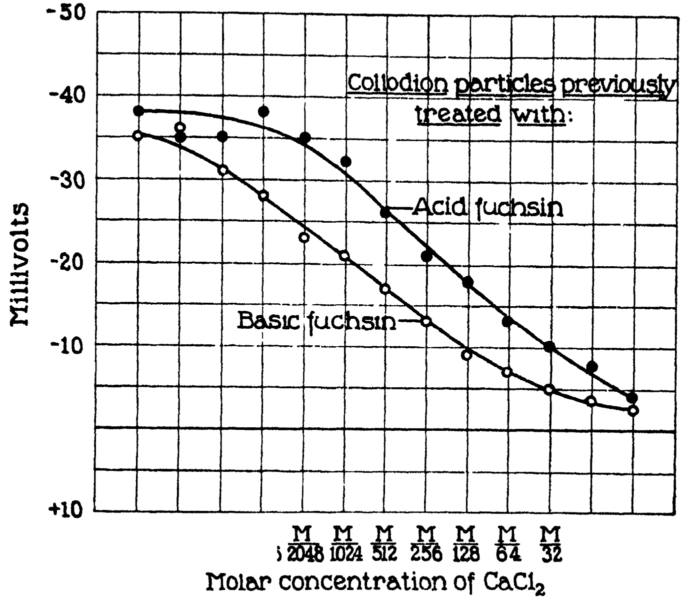

FIG. 6. Proof that the film formed on collodion particles by a previous treatment with dye cations (basic fuchsin) increases the depressing effect of $CaCl_2$ on the P.D., while a previous treatment with dye anions (acid fuchsin) has no such effect.

IV.

The influence of protein salts on the cataphoretic P.D. of collodion is of special interest. The isoelectric point of crystalline egg albumin is at pH 4.8, and it was shown in a preceding publication that a protein film is formed on the surface of collodion particles kept over night in a weak solution of isoelectric albumin.[5] These albumin-covered collodion particles were separated from the protein solution with the centrifuge and were then transferred into water of different pH to test the effect of electrolytes on their cataphoretic P.D. It was found

[5] Loeb, J., *J. Gen. Physiol.*, 1922–23, v, 479.

that at pH 4.8 or below, electrolytes influenced the particles as if they consisted of egg albumin, while for pH > 4.8, *i.e.* on the alkaline side of the isoelectric point of albumin, the particles behaved as if the albumin had been dissolved and as if only uncoated collodion particles were left. In the light of the experiments with dyes these results seem clear. At a pH above 4.8 the albumin forms only anions and these are incapable of being bound chemically by the collodion particles, while on the acid side of the isoelectric point albumin ions exist in the form of cations capable of being bound by the collodion and hence forming a durable film on the surface of the particles. That the isoelectric albumin is also bound at the surface of the collodion particles is of significance for the theory of the nature of the forces by which albumin is held by collodion, but this problem will not be considered in this paper. On the basis of these experiments it was to be expected that at a pH above 4.8, solutions of crystalline egg albumin should influence the cataphoretic P.D. of collodion particles similarly to solutions of acid dyes or of ordinary salts; while at a pH of 4.8, or below 4.8, the cataphoretic P.D. should be depressed in low concentrations of the protein.

Solutions of crystalline egg albumin varying from 1/500,000 of 1 per cent to 1 per cent were prepared at five different pH; namely, 11.0, 5.8, 4.8 (isoelectric point), 4.0, and 3.0, and the cataphoretic P.D. of collodion particles in these solutions was measured immediately (*i.e.* within less than 5 minutes after the collodion particles had been put into the solutions). Only the experiments with concentrations up to 1/16 per cent could be used without making a correction for the increase in the viscosity due to the protein in the calculation of the cataphoretic P.D. from the velocity of migration. When the concentrations of the protein exceeded 1/16 per cent, the influence of the increased viscosity had to be taken into consideration. The results of the experiments are given in Fig. 7. At pH 11.0 and 5.8 the addition of albumin did not alter the cataphoretic P.D. of the collodion particles at all. To judge from the experiments with acid dyes, the sodium albuminate could only have acted in this case like NaCl or Na_2SO_4, causing an initial rise of the P.D., but on account of the enormously high molecular weight of the albumin molecule—about 34,000 according to Sörensen—even a 1/16 per cent solution of sodium albuminate could have caused no increase in the P.D. at pH 5.8 or 11.0.

At pH 4.0 and 3.0, where the albumin forms a durable film on the surface of the collodion particles, the negative charge of the collodion particles is always annihilated at a low concentration of the albumin, 1/32,000 and 1/65,000 of 1 per cent respectively, and when the concentration of albumin rises above this value, the particles become

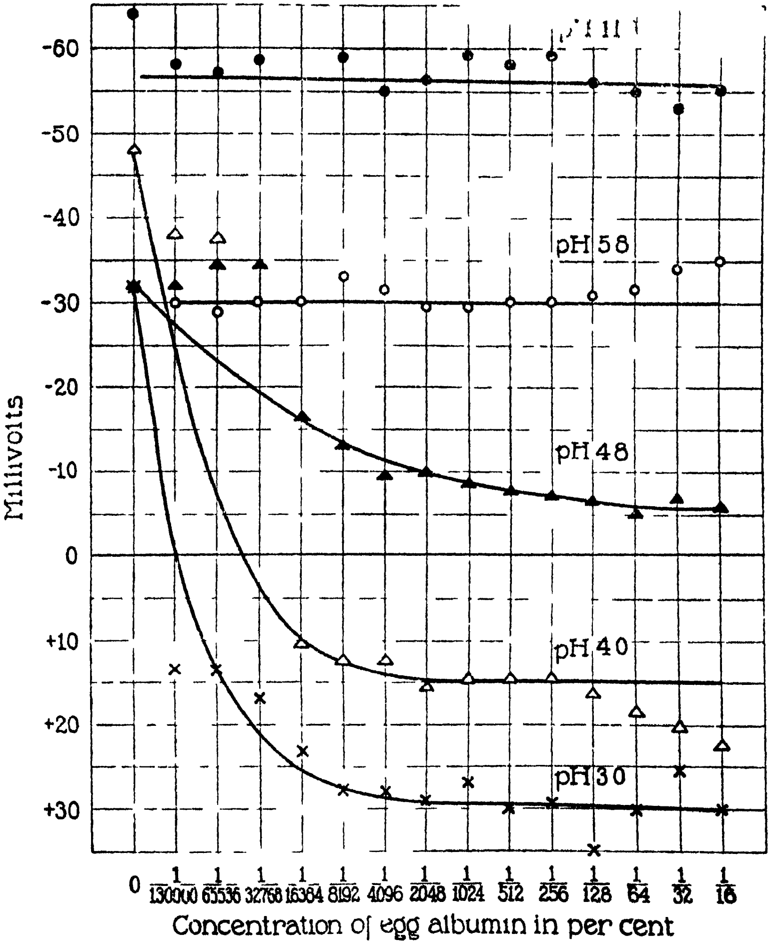

FIG. 7. The cataphoretic P D of the negatively charged particles of collodion is depressed by low concentrations of egg albumin of pH 4.8 or below but is not affected by egg albumin at pH 5 8 or 11.0. Low concentrations of egg albumin (1/130,000 and 1/32,000 of 1 per cent respectively) reverse the sign of the P.D. at pH 3.0 and 4 0 At pH 4 8, 4 0, and 3.0 where albumin influences the P.D. it forms a film on the collodion particles.

positively charged. The molecular concentration of albumin where this reversal of the sign of charge of the collodion particle occurs must therefore be in the neighborhood of 10^{-9} M. This is an order of magnitude which possibly approaches that of the efficiency of proteins in immunity and anaphylaxis.

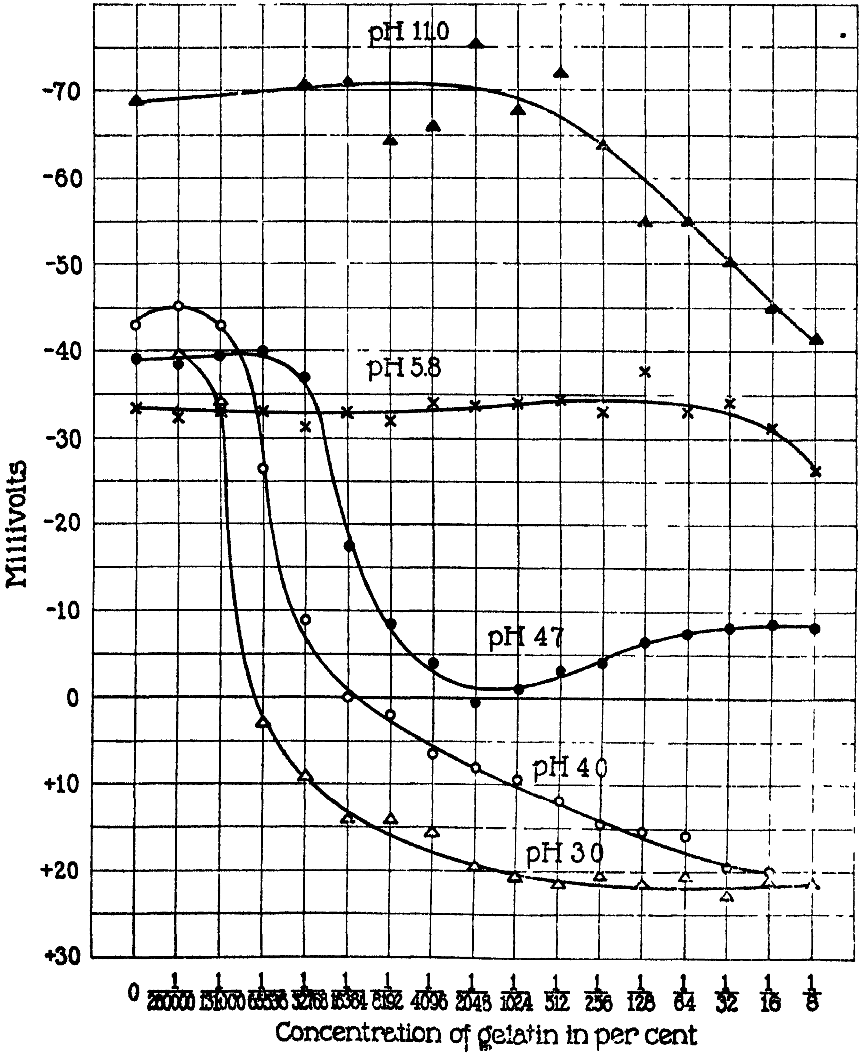

FIG. 8. The influence of low concentrations of gelatin on the cataphoretic P.D. of collodion particles is similar to that of egg albumin in Fig. 7.

Similar experiments were made with solutions of gelatin and it was found that gelatin behaves like albumin, as Fig. 8 shows. The negative charge of collodion particles was reversed in solutions of gelatin chloride of pH 3.0 when the concentration of the gelatin was 1/60,000 of 1 per cent; and at pH 4.0 when the concentration of the gelatin was between 1/8,000 and 1/16,000 of 1 per cent. At the isoelectric point of gelatin the P.D. of the collodion particles was brought to zero at a concentration of about 1/2,000 of 1 per cent, but no reversal of the sign of charge occurred.

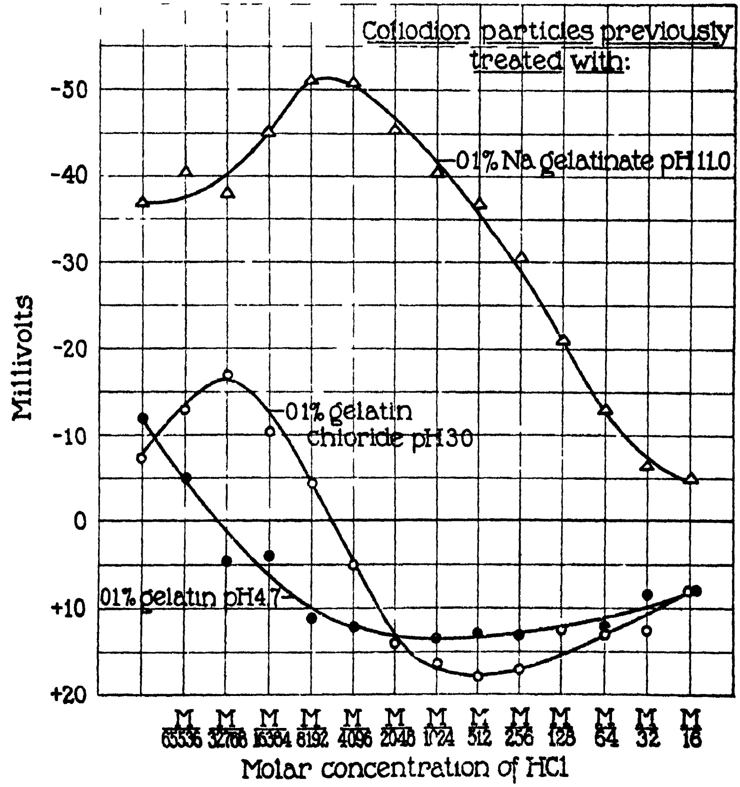

FIG. 9. Proof that gelatin forms a durable film on collodion particles only when it is isoelectric or on the acid side of its isoelectric point.

When collodion particles were left overnight in a 1/10 per cent sodium gelatinate solution of pH 11.0 and then separated from the protein solution by the centrifuge, they behaved cataphoretically like particles not treated with proteins; while when the collodion particles were left overnight in a 1/10 per cent solution of isoelectric gelatin (pH 4.7) or of gelatin chloride at pH 3.0, they behaved after being separated from the protein solution with the centrifuge like gelatin particles. This difference could be demonstrated by experiments on the influence of acids on the cataphoretic P.D. of these collodion particles (Fig 9). The upper curve in Fig. 9 shows that when the particles had previously been treated with Na gelatinate of pH 11.0 the addition of acid caused no reversal; if there had been a film of gelatin on the collodion particles they would have become

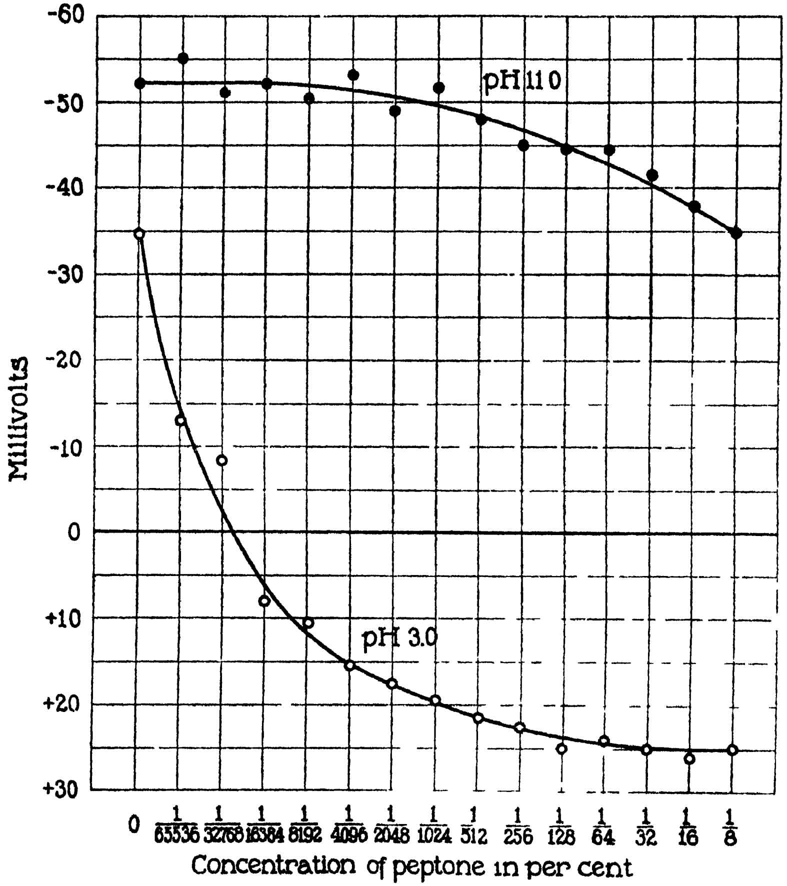

FIG. 10. Peptone acts similarly to albumin. See legend of Fig. 7.

positive under the influence of acid. Such a reversal was observed when the particles had been treated with isoelectric gelatin or with gelatin chloride (the two lower curves in Fig. 9).

V.

It was then intended to find out whether or not hydrolyzed proteins and amino-acids could still act like a protein on the cataphoretic P.D. Witte's peptone acted like a protein though the greater part of the original protein used for this preparation is hydrolyzed. Migration experiments showed that the isoelectric point of Witte's peptone is near pH 4.3. At pH 11.0 the peptone must have existed as anions, while at pH 3.0 it must have existed as cations. Filtered solutions of Witte's peptone containing from 1/65,000 to 1/8 of 1 gm. dry weight of peptone in 100 cc. of water were prepared at pH 11.0 and 3.0. Particles of collodion were put into these solutions and the rate of their cataphoretic migration was determined. Fig. 10 shows that at pH 11.0 the peptone had practically no effect on the cataphoretic P.D., while when the pH was 3.0 a solution of peptone of 1/32,000 of 1 per cent reversed the sign of migration of the particles.

When particles of collodion were kept overnight in a 0.5 per cent solution of isoelectric peptone and then separated from the peptone solution by centrifuging, they behaved in aqueous solutions of different salts (NaCl, $CaCl_2$, $LaCl_3$, Na_2SO_4, and $Na_4Fe(CN)_6$) like protein particles, the cataphoretic curves being like those described in the case of casein, denatured egg albumin, etc., thus showing that peptone or the non-hydrolyzed protein left in peptone forms a durable (not easily reversible) film on the surface of collodion particles. This was also corroborated by experiments on anomalous osmosis.

VI.

When, however, experiments were made with solutions of salts of amino-acids like alanine, tyrosine, or leucine, it was found that they always acted like ordinary crystalloidal salts. Since alanine is very soluble, it was possible to work with solutions as high as 1/16 gram molecular. Such solutions of alanine were prepared at three different pH, 3.0, 5.8, and 11.0. Collodion particles were put into the alanine solutions and their rate of migration was measured immediately. At pH 3.0 the alanine existed as cations; if alanine acted like albumin or gelatin or peptone, it should have reversed the negative charge of the collodion particles.

Fig. 11 shows that no such reversal occurred. Without salt the P.D. of the collodion particles was about 32 millivolts at pH 5.8. Nothing changed until the concentration of alanine exceeded M/1,024 when the P.D. dropped. In M/32 the P.D. of the particles was still negative (about 11 millivolts). Hence alanine chloride acted like NaCl but not like a basic dye or protein chloride on the cataphoretic P.D. of the collodion particles.

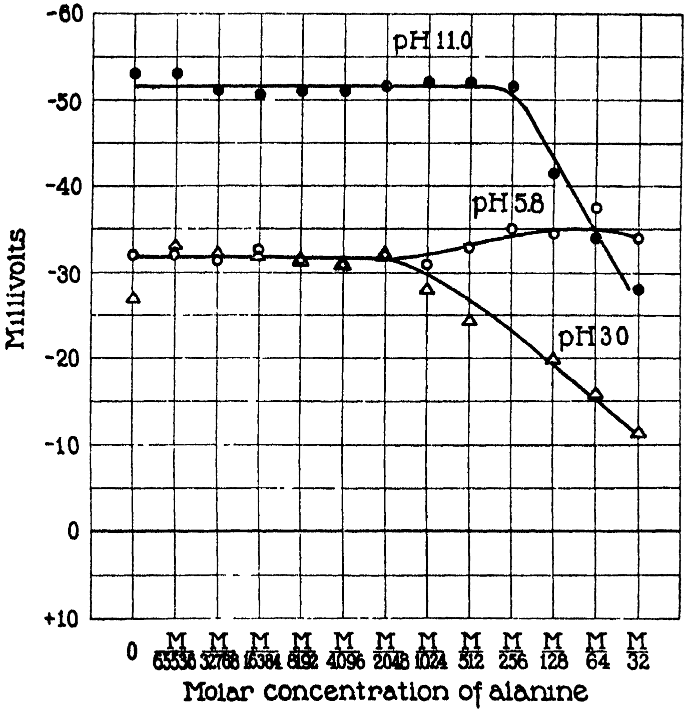

FIG. 11. Alanine acts on the cataphoretic P.D. like ordinary salts of the type NaCl.

At pH 5.8 the alanine is practically non-ionized and hence should leave the cataphoretic P.D. unchanged. Fig. 11 shows that this is true within the limits of the accuracy of the experiments. At pH 11.0 the sodium salt of alanine acts as any other salt, *e.g.* NaCl, would at the same pH on the cataphoretic P.D. of collodion particles, depressing the P.D. when the concentration of sodium alaninate exceeds M/256. There is no rise of the P.D. when the concentration of Na alaninate is

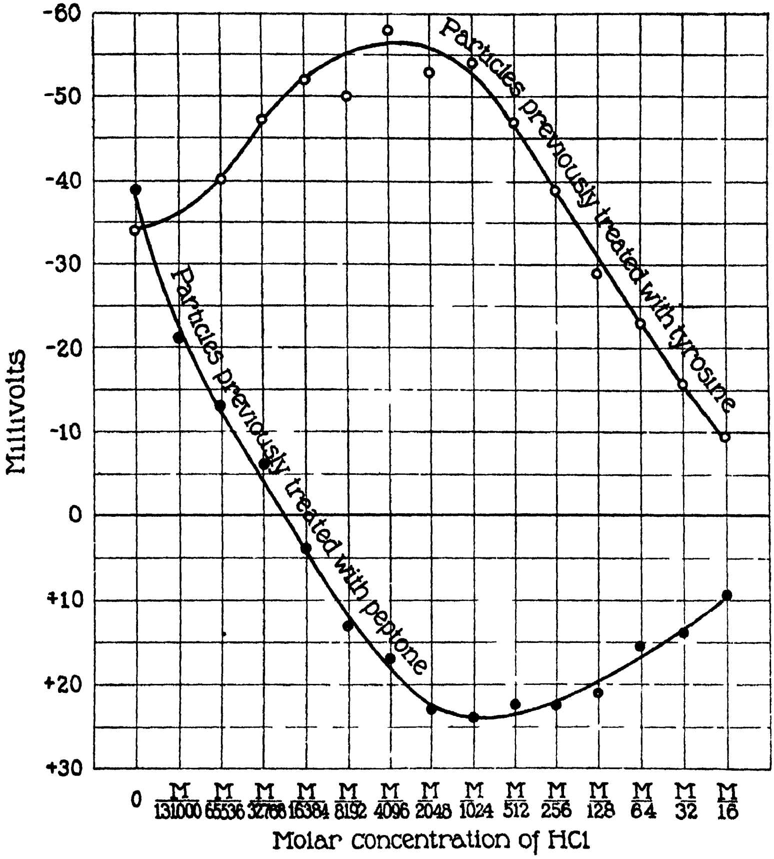

FIG. 12. Proof that amino-acids form no durable film on collodion particles. HCl of different concentrations affects the P.D. of collodion particles kept overnight in 1/25 per cent solution of tyrosine in the same way as if the particles had not been treated; while particles treated overnight in a solution of isoelectric peptone are rendered positive when the pH of the HCl solution falls below that of the isoelectric point of peptone (4.3).

below this value for the reason that at pH 11.0 the P.D. is already over 50 millivolts if no salt is added, so that salts can raise it only slightly at pH 11.0. NaCl acts in the same way on the cataphoretic P.D. of collodion particles as does Na alaninate.

The experiments with amino-acids seem of crucial importance since they show the difference between the action of ions which form durable films on the collodion particles and hence are attracted into the enveloping film by forces inherent in the collodion and of ions which do not form durable films. The amino-acid salts no longer act like proteins or peptone on the collodion particles but like ordinary crystalloidal salts. In order to make sure that the amino-acids do not form any durable film on the collodion particles, such particles were left overnight in 1/25 per cent solutions of tyrosine or 1/100 per cent solutions of leucine at pH 5.8 (near their isoelectric point). The next day they were separated from the solution of amino-acid with the aid of a centrifuge and the influence of different concentrations of HCl on the cataphoretic P.D. of the particles was tested. The upper curve in Fig. 12 gives the result, showing that HCl influenced the P.D. of these particles in the same way as if they had not been treated with tyrosine. If there had been any durable film of tyrosine on the particles, a low concentration of acid, *e.g.* that of the isoelectric point of tyrosine, should have caused a reversal of the sign of charge of the particle, which was not the case. When the particles were left overnight in *peptone*, HCl reversed the sign of charge of the particles already in a concentration of between M/32,000 and M/16,000, *i.e.* when the pH was that of the isoelectric point of peptone (lower curve in Fig. 12). This leaves no doubt that amino-acids form no durable film on the surface of the collodion particles. It was found that leucine also acts like tyrosine.

VII.

Since McTaggart was able to show that gas bubbles, which are naturally negatively charged, assume a positive charge in a solution of $LaCl_3$, it is obvious that the forces inherent in the aqueous solution itself can cause an excess of positive ions in the enveloping film. This does, however, not exclude the possibility that La (and Th) ions are also attracted chemically by the collodion particle. The only way of testing this was to find out whether $LaCl_3$ (or $ThCl_4$) acts like basic dyes or like protein chlorides, forming durable films which change the cataphoretic P.D. of collodion particles as do the films of basic fuchsin or of proteins. If durable films are formed on the collodion particles by $LaCl_3$, it would prove that ordinary salts are dragged into the enveloping film by the chemical forces inherent in the collodion particles; while when no durable film is formed such an attraction is not absolutely disproved.

Particles of collodion were kept overnight in 50 cc. of M/8 $LaCl_3$ at pH 3.0 and at pH 5.8. The particles were then separated from the solution with the centrifuge and then washed twice with distilled water of pH 5.8 and separated by the centrifuge from this water in order to separate the particles completely from the $LaCl_3$ solution, without removing a possible film of salt formed on the particles. The influence of various concentrations of NaCl on the cataphoretic velocity of the particles was then measured and compared with the influence of NaCl on collodion particles not previously treated with $LaCl_3$. The pH in all these velocity measurements was 5.8.

It was found that the influence of NaCl on the P.D. of the particles previously treated with $LaCl_3$ was the same as that on particles not treated with salt. There is then thus far no indication that ordinary salts like $LaCl_3$ form durable films on the surface of collodion particles.

VIII.

Solid particles suspended in water which are not ionized (and this is possibly true for collodion particles) can only move in an electrical field if they attract ions from the water or if ions are forced into the enveloping film (which adheres to the particle and moves with it) by forces inherent entirely in the water. The experiments contained in this paper show that both cases may occur. Proteins and basic dyes are attracted chemically by the particles, since both substances form durable films on the surface of the particle. The protein ions as well as the dye cations contribute to the electric charge of the enveloping film adhering to and moving with the collodion particles.

The question arises whether not all the charges of the collodion particles are due to such an attraction of the ions of ordinary electrolytes by the collodion particles. Our experiments show that this is probably not the case, for the following reason. The collodion particles are negatively charged in water and the addition of ordinary electrolytes of the type HCl, H_2SO_4, NaOH, NaCl, Na_2SO_4, $Na_4Fe(CN)_6$, and $CaCl_2$ increases the negative charge of the particles as long as the concentration of the electrolyte is low. If this phenomenon were due to a chemical attraction between particle and solute, it would mean that the collodion particle attracts anions but not cations. Such an assumption is, however, refuted by the experiments with dyes and proteins which show that only dye cations and protein cations are attracted but not protein anions. In all probability the forces of attraction in this case are not ionic at all since isoelectric protein also forms durable films with collodion particles. There remains then only the other possibility that the negative charge of collodion particles in water and the increase of this negative charge through the addition of low concentrations of electrolytes is due to forces inherent in the water, by which the extreme surface film of water has an excess of anions.

If this reasoning is correct, it leads to several interesting conclusions concerning the orientation of molecules and ions in the surface of the water. It is possible that these forces cause a definite orientation of the water molecules themselves of such a nature that the oxygen molecules of the water are turned outside, while both hydrogen ions are turned inwards. A small fraction of these molecules of water are dissociated electrolytically, so that as a consequence of the orientation of the water molecules the film of water enveloping the collodion particles and moving with them has an excess of anions. When a salt or alkali with monovalent cation is added to the water, the molecules of the solute at or near the surface will also be oriented in such a way that their anions are turned towards the free surface of the water (or towards the collodion particle), while the cations are turned inwards.

This tendency of the cation to be driven farther away from the interface and deeper into the water diminishes apparently with its increasing valency. Thus in the case of salts like $LaCl_3$ or $ThCl_4$ we must assume that it has disappeared completely or that it is even reversed. McTaggart has shown that low concentrations of $LaCl_3$ and still lower concentrations of $Th(NO_3)_4$ suffice to render gas bubbles positive in cataphoresis. This means that the ions of such salts no longer orient themselves in such a way as to turn their anions to the free surface of the water. Their orientation has ceased or may be even reversed. In this case only the forces inherent in the water itself are responsible for the molecular orientation.

In the case of electrolytes with monovalent cation the tendency of the anion to go in the enveloping film of the water may increase with the valency of the anion, though this is not certain.

When an electrolyte is added which forms a durable film on the surface of the particle, the situation is entirely different. In this case the dye cation or the protein cation of the film furnishes part of the charge of the enveloping film, while the other part is furnished by the excess of anions driven into the film by the forces inherent in the water itself.

We do not know at present the thickness of the enveloping film of water which adheres to and moves with the suspended particle; *i.e.*, whether this film is only one molecule deep or whether it is several molecules in thickness. Since ordinary electrolytes like NaCl, Na_2SO_4, etc., raise the surface tension of the water it is possible that their ions do not reach the outermost layer of the water. The question then arises how the molecules of a salt can influence the cataphoretic P.D. We must, in this case, also assume that the molecules of a salt are oriented in the surface layer but that the orientation occurs in a layer beneath the outermost surface stratum. Such an assumption was made by Lenard on the basis of his waterfall experiments, which led him to the conclusion that the outermost surface consists of H_2O molecules and that if salts are dissolved in the water, the cations are nearer to the surface than the anions. Since the addition of little salt increases the negative charge of collodion particles in cataphoresis, it would follow that as long as the concentration of a salt is small and the cations are only monovalent, the anions are nearer the interface than the cations; while with higher concentrations of the salts or with higher valency of the cation this difference disappears or is reversed.

The measurements of the rate of migration of the particles were carried out by the writer's assistant, Mr. M. Kunitz.

SUMMARY AND CONCLUSIONS.

1. The cataphoretic P.D. of suspended particles is assumed to be due to an excess in the concentration of one kind of a pair of oppositely charged ions in the film of water enveloping the particles and this

excess is generally ascribed to a preferential adsorption of this kind of ions by the particle. The term adsorption fails, however, to distinguish between the two kinds of forces which can bring about such an unequal distribution of ions between the enveloping film and the opposite film of the electrical double layer, namely, forces inherent in the water itself and forces inherent in the particle (*e.g.* chemical attraction between particle and adsorbed ions).

2. It had been shown in a preceding paper that collodion particles suspended in an aqueous solution of an ordinary electrolyte like NaCl, Na_2SO_4, $Na_4Fe(CN)_6$, $CaCl_2$, HCl, H_2SO_4, or NaOH are always negatively charged, and that the addition of these electrolytes increases the negative charge as long as their concentration is below M/1,000 until a certain maximal P.D. is reached. Hence no matter whether acid, alkali, or a neutral salt is added, the concentration of anions must always be greater in the film enveloping the collodion particles than in the opposite film of the electrical double layer, and the reverse is true for the concentration of cations. This might suggest that the collodion particles, on account of their chemical constitution, attract anions with a greater force than cations, but such an assumption is rendered difficult in view of the following facts.

3. Experiments with dyes show that at pH 5,8 collodion particles are stained by basic dyes (*i.e.* dye cations) but not by acid dyes (*i.e.* dye anions), and that solutions of basic dyes are at pH 5.8 more readily decolorized by particles of collodion than acid dyes. It is also shown in this paper that crystalline egg albumin, gelatin, and Witte's peptone form durable films on collodion only when the protein exists in the form of a cation or when it is isoelectric, but not when it exists in the form of an anion (*i.e.* on the alkaline side of its isoelectric point). Hence if any ions of dyes or proteins are permanently bound at the surface of collodion particles through forces inherent in the collodion they are cations but not anions. The fact that isoelectric proteins form durable films on collodion particles suggests, that the forces responsible for this combination are not ionic.

4. It is shown that salts of dyes or proteins, the cations of which are capable of forming durable films on the surface of the collodion, influence the cataphoretic P.D. of the collodion particles in a way entirely different from that of any other salts inasmuch as surprisingly

low concentrations of salts, the cation of which is a dye or a protein, render the negatively charged collodion particles positive. Crystalline egg albumin and gelatin have such an effect even in concentrations of 1/130,000 or 1/65,000 of 1 per cent, *i.e.* in a probable molar concentration of about 10^{-9}.

5. Salts in which the dye or protein is an anion have no such effect but act like salts of the type of NaCl or Na_2SO_4 on the cataphoretic P.D. of collodion particles.

6. Amino-acids do not form durable films on the surface of collodion particles at any pH and the salts of amino-acids influence their cataphoretic P.D. in the same way as NaCl but not in the same way as proteins or dyes, regardless of whether the amino-acid ion is a cation or an anion.

7. Ordinary salts like $LaCl_3$ also fail to form a durable film on the surface of collodion particles.

8. Until evidence to the contrary is furnished, these facts seem to suggest that the increase of the negative charge of the collodion particles caused by the addition of low concentrations of ordinary electrolytes is chiefly if not entirely due to forces inherent in the aqueous solution but to a less extent, if at all, due to an attraction of the anions of the electrolyte by forces inherent in the collodion particles.

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CONDUCTIVITY TITRATION OF GELATIN SOLUTIONS WITH ACIDS.

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(Received for publication, October 4, 1923.)

The combination of proteins with acids has been studied by Loeb[1] and others largely by means of hydrogen electrode measurements. The present work constitutes an attempt to apply the method of conductivity titration to the determination of the combining capacity of a protein for acids.

The first work which indicated that the measurement of electrical conductivity could be used in volumetric analysis seems to have been done by Kohlrausch[2] in 1885, and since then the method has been given in standard text-books.[3] Recently the method has been extensively used by Kolthoff,[4] who has outlined the theory on which it depends. In the neutralization of a weak base by a strong acid, the conductivity at first increases because of the formation of a highly ionized salt, and since at first practically all of the added acid is used up to form the salt, the conductivity increases as a linear function of the amount of acid. As more acid is added, the formation of the salt is no longer complete on account of hydrolysis, and the increase in conductivity becomes faster, due to the presence of the highly mobile hydrogen ion. Finally, after enough acid has been added to combine with all the base and to repress the hydrolysis, the conductivity increases much more rapidly on the addition of further acid, since practically all the added hydrogen ion remains free; and here again the conductivity increases as a linear function of the added acid. The point of neutralization is obtained by plotting the conductivity as a function of the quantity of acid added and extrapolating the two linear portions of the curve until they intersect. The shape of all the curves obtained with gelatin is similar to that of Fig. 1. Kolthoff[4] obtained curves of like shape by neutralizing phenol or boric acid with sodium hydroxide, and by neutralizing urotropin (hexamethylenetetramine) with hydrochloric acid. He thus showed that the method was applicable to the neutralization of a weak acid or base with a strong base or acid.

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[1] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, Chapter IV.

[2] Kohlrausch, F., *Ann. Physik u. Chem.*, 1885, xxvi, 225.

[3] Kohlrausch, F., and Holborn, L., Das Leitvermögen der Elektrolyte, Leipsic and Berlin, 2nd edition, 1916, 136. Findlay, A., Practical physical chemistry, London and New York, 3rd edition, 1914, 199.

[4] Kolthoff, I. M., *Z. anorg. Chem.*, 1920, cxi, 1, 28, 97; cxii, 155, 165, 172, 187, 196.

In applying the method to the neutralization of a protein it is necessary to consider the fact that proteins are amphoteric electrolytes. In some preliminary experiments in which gelatin solutions were first treated with sodium hydroxide and the resulting solutions titrated with hydrochloric acid, it was found that the slope of the conductivity curve exhibited practically no change at the isoelectric point. Therefore if the original gelatin solution is not at the isoelectric point it is necessary to make a correction for this in calculating the combining capacity of gelatin for acid.

The following experimental procedure was adopted. A measured volume of a solution containing a known weight of gelatin was placed in the conductivity cell, in a water bath at 33°C. ± 0.02°. The cell used was of the Freas type, holding about 130 cc. Its cell constant was not determined, since only relative conductivities were needed for this work. The conductivity was measured in the usual way with a Kohlrausch bridge and resistance box, using a small induction coil and a telephone. Standard acid was added in small amounts from a calibrated 10 cc. or 2 cc. burette, and after each addition the conductivity was measured. The results were plotted as in Fig. 1, the abscissæ being cc. of acid and the ordinates being proportional to the specific conductivities. From the intersection of the two straight lines the end-point was obtained. The pH was measured of a separate sample of each original gelatin solution, using hydrogen electrodes at 33° with a saturated KCl junction, taking as a standard the value 1.037 for the pH of M/10 HCl. The values for the end-point were corrected to correspond with an initial pH of 4.70 by adding or sub-

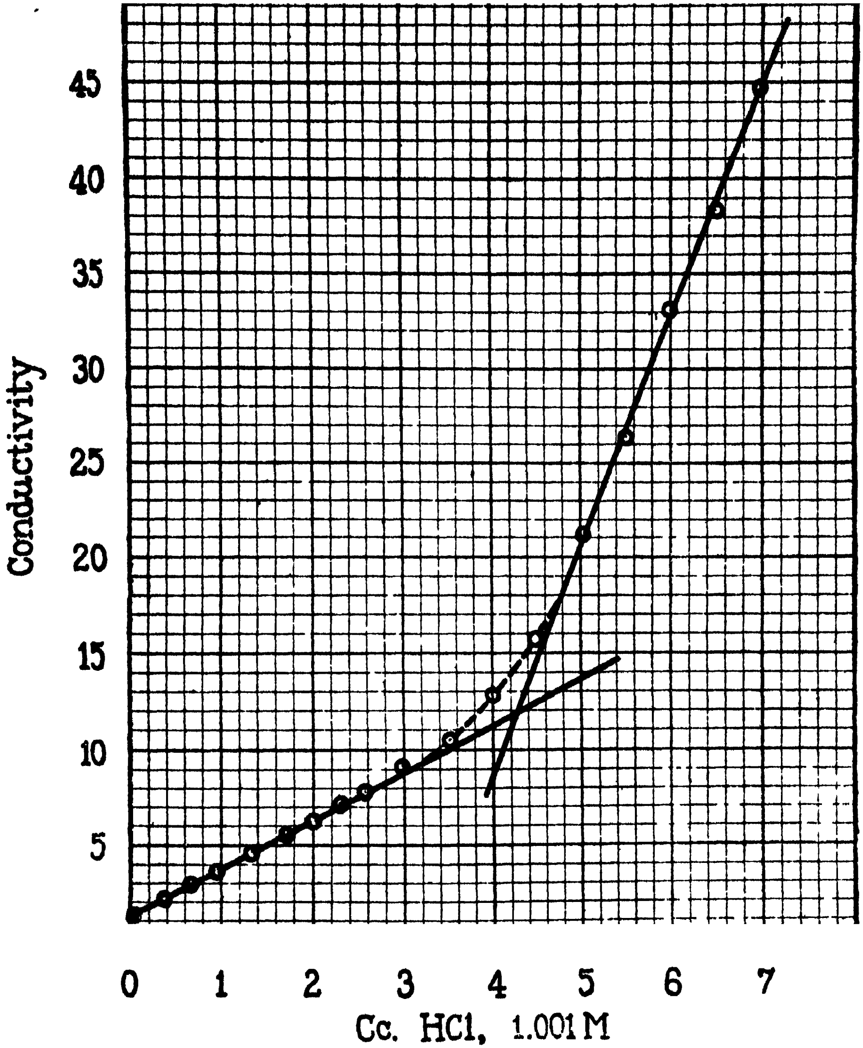

FIG. 1. Conductivity titration of gelatin with hydrochloric acid. The lower straight line represents practically complete formation of gelatin chloride from the HCl added. The curved dotted line represents the increase in conductivity due to hydrolysis of gelatin chloride. The upper straight line represents the increase in conductivity due to excess HCl, after the hydrolysis has been repressed. The intersection of the two straight lines is the apparent point of equivalence, which must be corrected if the initial pH is not that of the isoelectric point.

tracting the amounts of acid indicated by the curve previously obtained[5] for the combination of gelatin with hydrochloric acid. This curve appears to be linear at the lower end, a change in pH from 4.78 to 4.25 corresponding to 2.0 cc. of M/10 HCl per gm. of gelatin. The results obtained with different concentrations and different lots of approximately isoelectric gelatin and hydrochloric and sulphuric acids are given in Table I. Experiment 4 is represented also in Fig. 1, which is typical of all the curves obtained. The end-points in Table I were obtained from curves plotted on a much larger scale than Fig. 1. The ordinates of Fig. 1 are 1,000 times the conductivities in reciprocal ohms obtained with the particular cell used.

TABLE I.

Conductivity Titration of Gelatin with Acids.

Experiment No.	Wt. of gelatin.	Initial volume.	Normality of acid.	End point, acid.	N/10 acid per gm. gelatin.	Initial pH	Correction to pH 4.70, N/10 acid.	N/10 acid equivalent to 1 gm. gelatin.
	A. Hydrochloric acid.							
	gm.	*cc.*		*cc.*	*cc.*		*cc.*	*cc.*
1	0.504	100	1.001	0.395	7.84	4.55	+0.57	8.41
2	1.815	90.7	1.001	1.58	8.71	4.75	−0.19	8.52
3	3.992	100	1.001	3.76	9.42	4.84	−0.53	8.89
4	4.915	100	1.001	4.27	8.69	4.74	−0.15	8.54
Average.								8.59
	B. Sulphuric acid.							
5	0.504	100	1.073	0.363	7.72	4.55	+0.57	8.29
6	1.822	94	1.073	1.42	8.36	4.73	−0.11	8.25
7	1.996	50	1.073	1.76	9.46	4.84	−0.53	8.93
8	4.915	100	1.073	4.08	8.91	4.74	−0.15	8.76
Average.								8.56

In order to determine whether this method of determining the end-point was affected by the change in volume due to the addition of the reagent, a trial calculation was made with the data of Experiment 8, in which a total of 7.5 cc. of acid was added to 100 cc. of gelatin solution. Assuming as an extreme case that the conductivity was inversely proportional to the volume, and that the quantity measured should have been the conductivity at a constant volume of 100 cc., a curve was constructed which indicated an end-point at 4.12 cc., while that obtained by neglecting the volume change was 4.08 cc. Therefore the change in volume could not have caused an error of much over 1 per cent.

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[5] Hitchcock, D. I., *J. Gen. Physiol.*, 1921–22, iv, 733.

While the results in Table I do not all agree within this error, yet the average of the figures for HCl, 8.59, is quite close to the average for H_2SO_4, 8.56. Roughly, then, the data indicate that 1 gm. of isoelectric gelatin can combine with a maximum of 8.6 cc. of N/10 HCl or H_2SO_4. The figure obtained from hydrogen electrode measurements alone, corrected for an initial pH of 4.70, was 8.9 cc. of N/10 HCl.[6]

A better check was obtained in the case of deaminized gelatin. The solution used was that described in a previous paper;[6] its pH was 4.0. A 25 cc. sample, containing 0.742 gm., was titrated with 1.000 N HCl, giving an end-point at 0.323 cc. Therefore 1 gm. was equivalent to 4.35 cc. N/10 HCl, no correction for the isoelectric point being required. The value obtained by the hydrogen electrode method was 4.4 cc.

SUMMARY.

Titrations have been made, by the conductivity method, of gelatin solutions with hydrochloric and sulphuric acids. The results indicate an end-point at about 8.6 cc. of N/10 acid per gm. of gelatin, or a combining weight of about 1,160. These results are in fair agreement with those previously obtained by the hydrogen electrode method. Better agreement between the two methods was found in the case of deaminized gelatin. The data are in accord with a purely chemical conception of the combination between protein and acid.

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[6] Hitchcock, D. I., *J. Gen. Physiol.*, 1923–24, vi, 95.

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THEORY OF REGENERATION BASED ON MASS ACTION. II.

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(Received for publication, September 4, 1923.)

I.

The polarity in the regeneration of an isolated piece of stem of *Bryophyllum calycinum* expresses itself by two characteristics which must be treated separately; namely, first, the fact that regeneration occurs only at the extreme ends, and, second, that the character of the regenerated organs is different at the opposite ends, shoots being formed at the most apical node and roots at the extreme basal end of the piece.[1] The second fact had been explained by Sachs on the assumption that the descending sap sent out by the leaf contains specific substances fit for root formation while the ascending sap sent out by the leaf contains specific substances for shoot formation. This explanation does not seem tenable any longer in view of the fact, first, that the sap of the leaf favors root and shoot formation in the same notch of a leaf, and second, that it can be shown that under proper conditions the descending sap favors also shoot formation in the stem.[2] The difference in the character of the regenerated organs at the opposite ends of a piece of stem must therefore have a different reason and it was suggested that the descending sap reaches primarily cells or tissues which can give rise to roots and not to shoots, while the ascending sap primarily reaches tissues which can give rise to shoots.[1] Differences in the chemical nature of the descending and ascending sap sent out by the leaf may or may not exist; if they exist they cannot be the cause of the different character of the organs formed at the opposite ends of the stem.

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[1] Loeb, J., *J. Gen. Physiol.*, 1921-22, iv, 447.

[2] Loeb, J., *J. Gen. Physiol.*, 1922-23, v, 831.

If by mutilation the descending sap from a leaf can be deflected from its path, it may give rise to shoots as was shown in the preceding paper.

That the regeneration in a piece of isolated stem of *Bryophyllum* is confined to the extreme ends of a piece of stem is, however, only a secondary phenomenon which demands an entirely different explanation than the fact that the character of the two regenerates is different. In a preceding paper it had already been shown that in the beginning, *i.e.*, during the first 8 or 10 days, at greenhouse temperature, the regeneration in a piece of stem does not possess that pronounced character, which is understood by polarity; namely, that roots are only formed at the extreme base and shoots only at the extreme apex. One of the writer's earlier experiments[3] may be mentioned to illustrate this fact.

Pieces of stem of *Bryophyllum calycinum* are cut out from vigorous plants and all leaves with the exception of the two leaves at the most basal node are removed from the pieces. The base of the stems below the leaves dips into water. In this case, air roots grow out from the nodes above the leaves before they grow from the base of the stem; and shoot formation commences not only in the most apical buds but also in the buds in the second or third node below the apex. Under these conditions there is almost no indication that the character of regeneration in the stem is polar.[3]

At greenhouse temperature this may all be observed within the first 8 or 10 days of the experiment, then the picture changes. At the base of the stems (dipping into water) roots now commence to appear and they grow more rapidly than the air roots which had appeared previously in the nodes of the stem. The air roots in the more apical nodes now begin to wilt and soon disappear. The more rapid growth of the basal roots dipping into water seems to suppress the further growth of the air roots which had previously formed. Likewise the shoots in the most apical node now commence to grow more rapidly than the anlagen for shoots in the nodes below the apex and the growth of these latter shoots now stops also. That feature of polarity which consists in the restriction of root formation to the extreme base and of shoot formation to the extreme apex of the piece of stem is not a primary but a secondary phenomenon. We will show that this same phenomenon can be demonstrated in a leaf and that it can be shown in this case that it is the expression of the following general rule in regeneration which the writer had already expressed in earlier publications; namely, that when in an isolated leaf (or in a piece of stem) the rate of growth of one type of organs is accelerated, all the sap will flow to these more rapidly growing organs, with the result that the growth of the competing organs will be suppressed. It is intended to furnish the quantitative proof for this rule.

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[3] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 687.

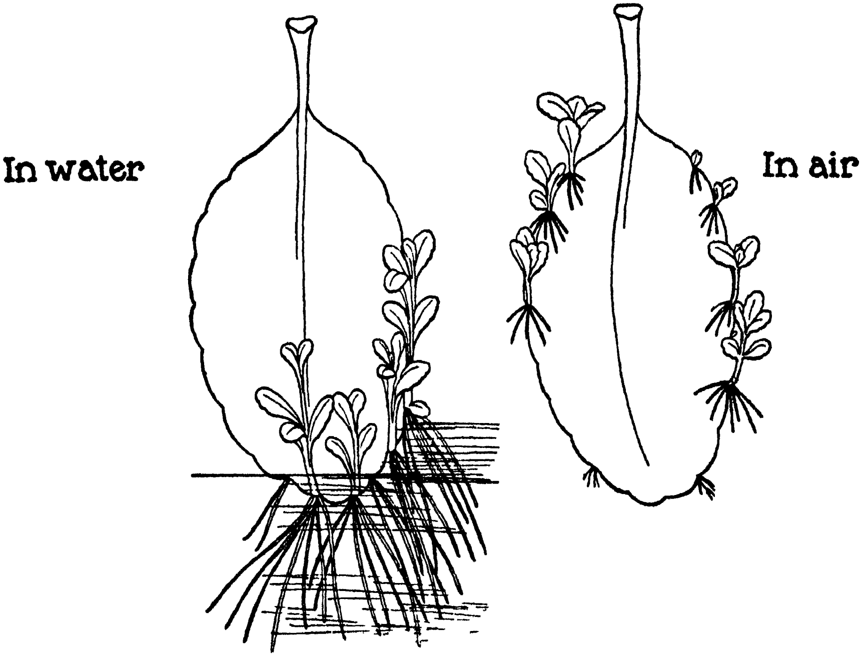

FIG. 1. Difference in the place of regeneration when the leaf is suspended entirely in air and when its apex dips into water.

When a leaf of *Bryophyllum* is suspended in moist air, shoots and roots will grow from the notches in the middle of the leaf or nearer the petiole where the leaf is more fleshy and where more sap is available than at the thin apex of the leaf (see Fig. 1, leaf marked "in air"). When, however, the apex of the leaf is dipped into water, while the rest is in air (Fig. 1, leaf marked "in water"), roots and shoots will grow out only from those notches of the apex which dip into the water or are close to the surface of the water. The other notches commence also to grow at first and tiny roots and tiny shoots may be formed; but as soon as the notches which dip into water commence to grow, all the growth of the shoots and roots in the other notches not in contact or in close proximity with water will cease. It can be shown that the reason for this inhibition is that the notches dipping into water grow more rapidly than the notches growing in air and that as a consequence of this more rapid growth a flow of the sap of the whole leaf towards the more rapidly growing notches is established. Thirteen pairs of sister leaves were used for the experiment, one leaf of each pair dipping with its apex into water while the sister leaf was suspended entirely in air. Table I shows that the mass of shoots and roots produced in the leaves dipping into water was greater than the mass of shoots and roots produced simultaneously and under equal conditions by the sister leaves suspended in air.

TABLE I.

	Dry weight of leaves	Dry weight of shoots	Dry weight of roots	1 gm dry weight of leaves produced	
				Shoots.	Roots
	gm.	*gm.*	*gm.*	*mg*	*mg*
13 leaves dipping in water .	1.943	0 524	0 123	270	63
13 leaves suspended in air . .	1 909	0.322	0 051	169	27

It is obvious that the leaves suspended in air form a much smaller quantity of dry weight of shoots and roots per gm. of dry weight of leaf during the same time and under the same conditions than the leaves dipping into water. Hence, if we accelerate the growth of some notches in the leaf, *e.g.*, by dipping them into water, we thereby inhibit the growth in the other notches.

That this inhibition is merely due to the fact that all the material available in the leaf now goes to the shoots in the apex can also be proven by quantitative experiments. The writer had shown in a previous publication[8] that if an isolated leaf is suspended vertically and sidewise in moist air (Fig. 2), *i.e.*, the middle rib being in a horizontal position, while the surface of the leaf is vertical (Fig. 2), shoots and roots develop with preference, and in most cases exclusively, on the lower edge of the leaf. This can be explained on the assumption that through the influence of gravity more liquid collects in the lower edge and that as a consequence the growth in these lower notches is accelerated, and as a further consequence a flow of material to the more rapidly growing lower notches is established, whereby all the material available in the leaf flows to the lower edge.

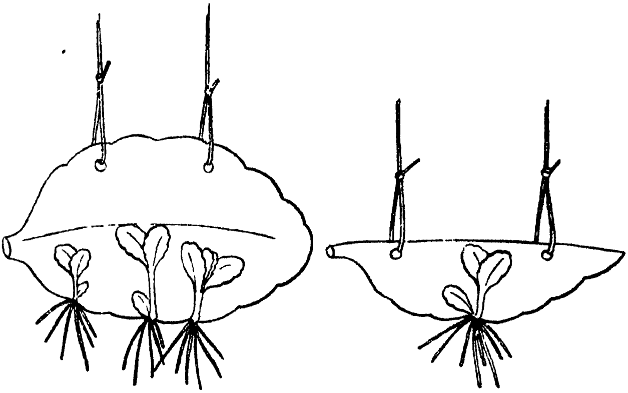

FIG. 2. Quantitative difference in regeneration when a whole leaf and a half leaf are suspended sidewise in air.

TABLE II.

	Dry weight of leaves	Dry weight of shoots.	1 gm. dry weight of leaves produced dry weight of shoots.
	gm.	*gm.*	*mg.*
13 whole leaves.	2.174	0.410	188
13 half sister leaves.	1.118	0.235	209

A number of pairs of sister leaves were suspended vertically and sidewise in air, one leaf of each pair remaining intact, while the upper half of the sister leaf was cut away (as shown in Fig. 2). The whole leaves formed practically twice as great a mass of shoots and roots on the lower edge as the half leaves and this difference is noticeable in Fig. 2. Thirteen whole leaves and their thirteen half sister leaves were chosen for comparison in Table II.

This proves that the excess regeneration in the *whole* leaves was produced by material furnished by both the lower and the upper half of the leaves, regeneration in the upper half being as a consequence impossible, since almost all the material available for regeneration in the upper half was consumed for regeneration in the lower half.

In the case just discussed the regeneration in the lower half of the leaf is accelerated since owing to the action of gravity the liquid in the leaf collects in the lower half. This happens only when the leaf is suspended sidewise in the air but not when it is suspended in the air with the apex down; as is shown by a comparison of the left -hand drawing in Fig. 2 with the right-hand drawing in Fig. 1. The reason for this difference is probably that the apex of the leaf is very thin in comparison with the fleshy middle part of the leaf so that under the influence of gravity liquid cannot collect as abundantly in the apex of the leaf as in the lateral parts.

II.

It had been shown in a preceding paper[1] that the mass law holds also for the regeneration of roots and shoots in a defoliated piece of stem of *Bryophyllum* when exposed to light. The dry weight of shoots and roots regenerated by such pieces in a given time under given conditions is approximately in proportion to the dry weight of the stem; and when a long stem of *e.g.* eight nodes is cut into eight pieces, each containing only one node, the total dry weight of the sixteen shoots produced by these pieces in a given time equals approximately the dry weight of the two apical shoots produced in the same time and under the same conditions by a stem of equal dry weight, not cut into smaller pieces. This shows that all the material available for regeneration in a larger piece of defoliated stem goes into the apical shoots and basal roots.

When a piece of stem is cut into as many pieces as there are nodes the shoots commence to grow out from the nodes regardless of the order in which they had originally been arranged in the stem; but in each piece the rate of growth corresponds approximately to the dry weight of the piece of stem, except in very old pieces of stem where not all material may be alive or in the very young pieces at the apex, where the axillary buds capable of growing into shoots may not yet have been properly developed.[1]

In the introduction to this note attention has been called to the fact that when regeneration begins in a piece of stem under the conditions described, regeneration is at first not markedly polar, since the first roots do not grow out at the base of the stem, but from the more apical nodes; and the anlagen for shoots do not grow out only in the apical node, but also below. The polar character becomes only established after about a week, when the roots at the extreme base begin to appear, and when the rate of growth of the most apical shoots suddenly exceeds that of the anlagen for the buds in the lower nodes. On the basis of the quantitative experiments on leaves described in this paper we must conclude that the more rapid growth of the most apical bud and of the basal roots which starts after about a week coincides with a collection of sap at the extreme ends of the piece of stem. This collection of sap caused by the block of the ascending sap at the apical node and of the descending sap at the base of the stem acts in a similar way as the collection of sap on the lower edge of a leaf suspended sidewise; namely, accelerating growth at the place where the sap collects. This acceleration of growth now influences the sap flow so that all the ascending sap goes to the apical node, and all the descending sap to the base, cutting off that supply of material for the rest of the stem which was available immediately after the operation.

SUMMARY.

1. Quantitative proof is furnished that all the material available for shoot and root formation in an isolated leaf of *Bryophyllum calycinum* flows to those notches where through the influence of gravity or by a more abundant supply of water growth is accelerated. As soon as the acceleration of growth in these notches commences, the growth of shoots and roots in the other notches which may already have started ceases.

2. It had been shown in a preceding paper that the regeneration of an isolated piece of stem may be and frequently is in the beginning not markedly polar, but that after some time the growth of all the roots except those at the base and of all the shoots except those at the apex is suppressed. This analogy with the behavior of regenera-

tion in a leaf in which the growth in one set of notches is accelerated, suggests that in an isolated stem a more rapid growth is favored at the extreme ends (probably by a block of the sap flow at the extreme ends) and that when this happens the total flow of ascending sap goes to the most apical buds and the total flow of the descending sap goes to the most basal roots. As soon as this occurs, the growth of the other roots and shoots is suppressed.

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THE INFLUENCE OF THE CHEMICAL NATURE OF SOLID PARTICLES ON THEIR CATAPHORETIC P.D. IN AQUEOUS SOLUTIONS.

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(Received for publication, August 20, 1923.)

I.

INTRODUCTION.

In order to arrive at a theory of the action of electrolytes on the formation of a double electrical layer—or, more correctly, on the value of the cataphoretic P.D.—between solid particles and aqueous solutions, it is necessary to know how this action is influenced by the nature of the suspended particle. In former papers by the writer, the effect of electrolytes on the cataphoretic P.D. was studied on proteins[1] and on collodion particles.[2] In this paper experiments on particles of mastic, Acheson's graphite (trade name "Aquadag," Size 5), which I owe to the kindness of Dr. Leonard Waldo, gold (prepared by Bredig's method in solution of N/10,000 HCl), ferric hydroxide, and calcium oxalate are added. To give a more complete picture experiments on collodion and protein are included.

In order to eliminate as much as possible the influence of H and OH ions, the experiments with salts were made in distilled water as near the point of neutrality as was conveniently possible. The distilled water used had a pH of about 5.8, resulting from an equilibrium with the CO_2 of the air of the laboratory. Stock solutions of the salts were prepared with a pH of 5.8 and these were diluted with distilled water of the same pH. Great care was taken to keep the pH of the solutions at 5.8, except in the case of solutions of $ThCl_4$ which were always more acid on account of the hydrolytic dissociation of the salt; and of solutions of acid and basic fuchsin, where the pH was not measured.

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[1] Loeb, J., *J. Gen. Physiol.*, 1922–23, v, 395.

[2] Loeb, J., *J. Gen. Physiol.*, 1922–23, v, 109; 1923-24, vi, 105.

It was also thought advisable to eliminate impurities as much as possible. Such impurities were contained in some cases in the stock solution of the suspended particles. This error was minimized by using as small a quantity of suspended particles as possible. A few drops of the stock suspension were put into 50 cc. of the salt solution, shaken, and allowed to stand for 20 minutes. When this is done, it is found that the P.D. of the particles in distilled water of pH 5.8 without the presence of salts is generally very small, rarely above 8 millivolts. Only when the particles are themselves electrolytes or when electrolytes are added does the P.D. rise. Statements in the literature that a high cataphoretic P.D. was observed in distilled water without electrolytes suggest the presence of electrolytes in the form of impurities, unless the particle itself was an electrolyte. The value of 26 millivolts for mastic particles in pure water at pH 5.8 in Fig. 2 is too high and unquestionably due to an impurity of some kind. A repetition of the experiments where such errors were excluded gave a much lower P.D., namely about 8 millivolts or less.

Although the P.D. of the particles in water at pH 5.8 is very low in the absence of electrolytes, the sign of charge of the particles at this low P.D. is of the greatest significance for the effect of the salt on the P.D. This effect is entirely different according to whether a particle is positively or negatively charged in pure water near the point of neutrality even if the P.D. be as low as 5 millivolts. We shall therefore designate as negative colloids, such particles; which at pH 5.8 are negatively charged in the absence of electrolytes; and as positive colloids, particles which are positively charged at pH 5.8 in the absence of electrolytes.

The method of determining the cataphoretic P.D. was the same as in the preceding papers, namely, the measurement of the rate of migration of isolated particles under the microscope in the way described by Ellis.[3] From the measurements of the velocity of migration the P.D. was calculated with the aid of the Helmholtz-Perrin formula. The apparatus used was that by Ellis as modified by Northrop,[4] with

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[3] Ellis, R., *Z. physik. Chem.*, 1911–12, lxxviii, 321; 1912, lxxx, 597.

[4] Northrop, J. H., *J. Gen. Physiol.*, 1921–22, iv, 629.

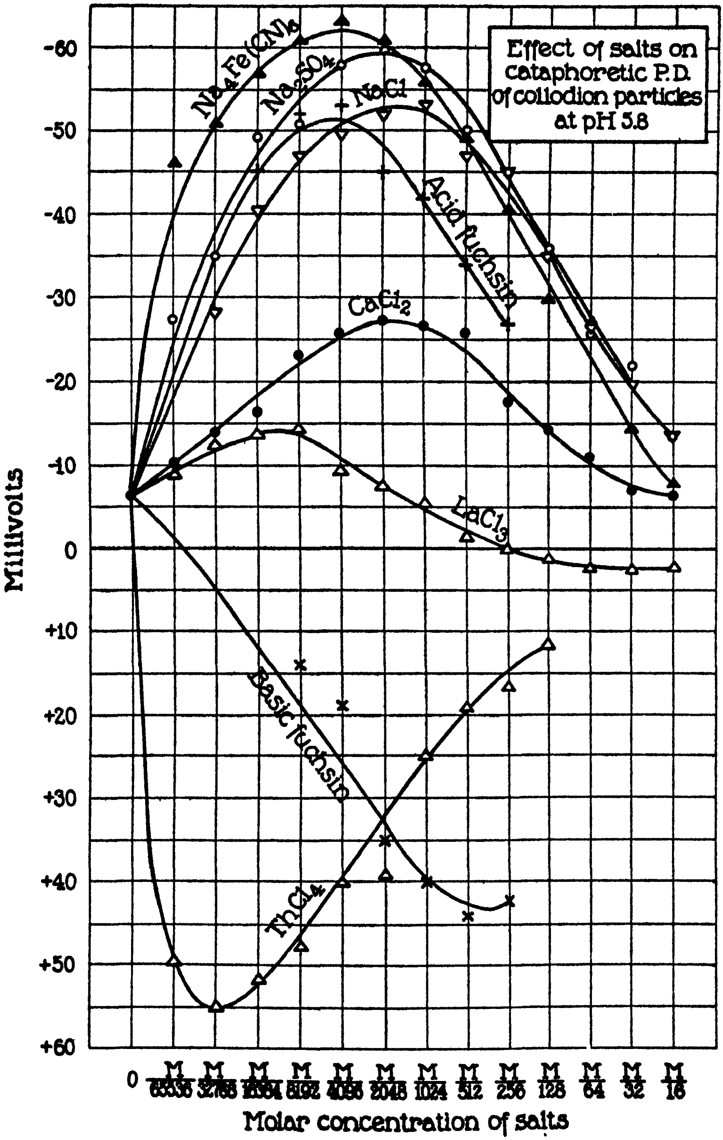

FIG. 1. Influence of salts on the cataphoretic P.D. between collodion particles and aqueous solutions near the point of neutrality, pH 5.8, (with the exception of the solutions of $ThCl_4$ and basic fuchsin). Abscissæ are the molar concentrations of salt solutions, ordinates, the cataphoretic P.D. The signs minus and plus refer to the sign of charge of the particles.

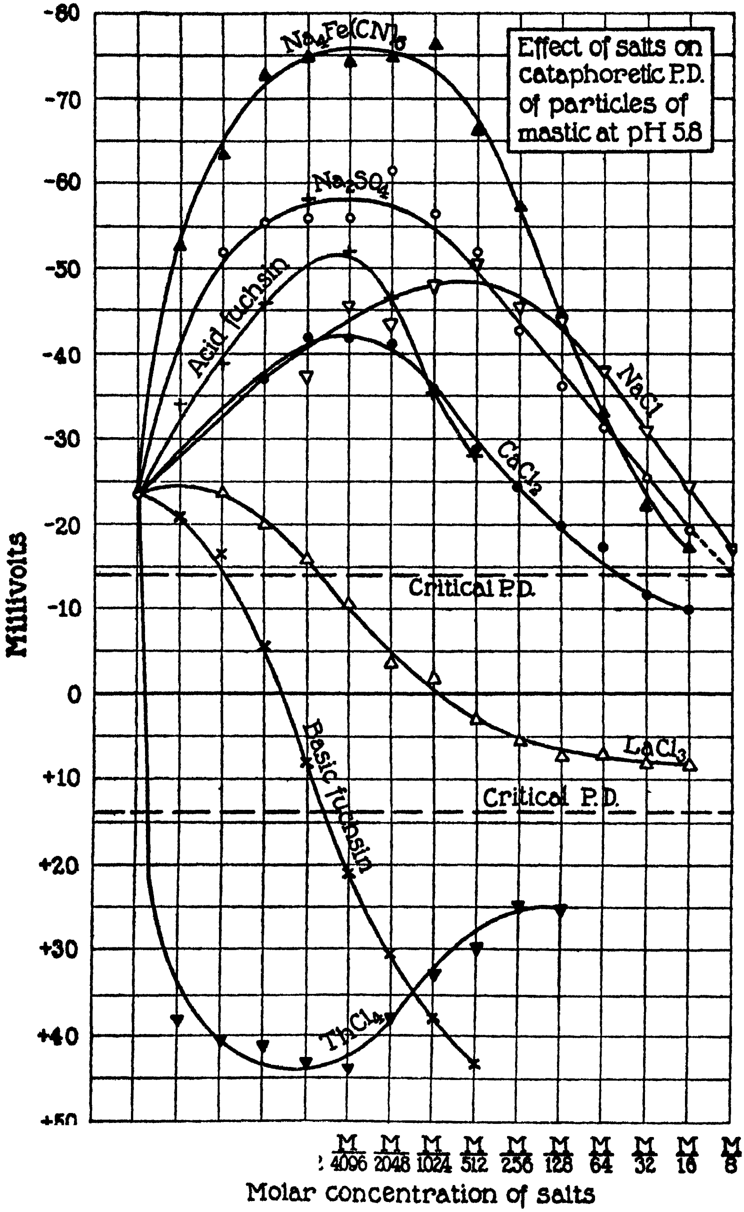

FIG. 2. Influence of salts on the cataphoretic P.D. of mastic particles near neutrality (pH 5.8). The line Critical P.D. indicates the value of P.D. below which the suspension ceases to be stable.

an additional modification introduced by Mr. Kunitz for using dark-field illumination.

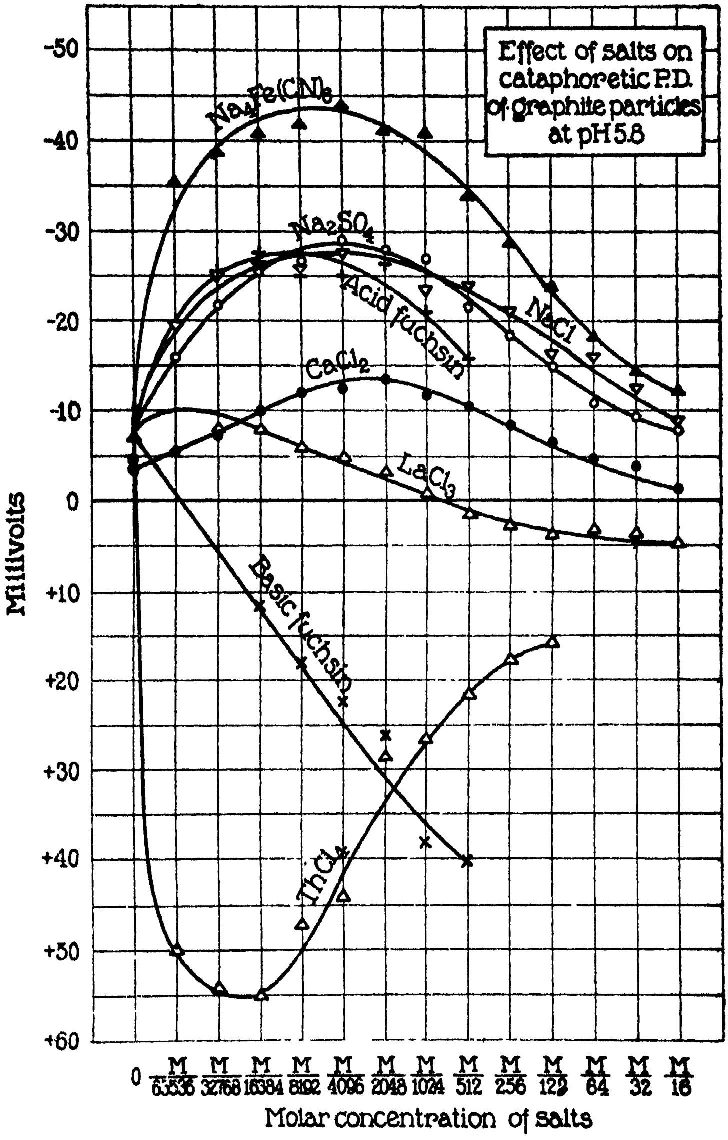

FIG. 3. Influence of salts on the cataphoretic P.D. of particles of Acheson's graphite (trade mark "Aquadag," Size 5) near neutrality, pH 5.8.

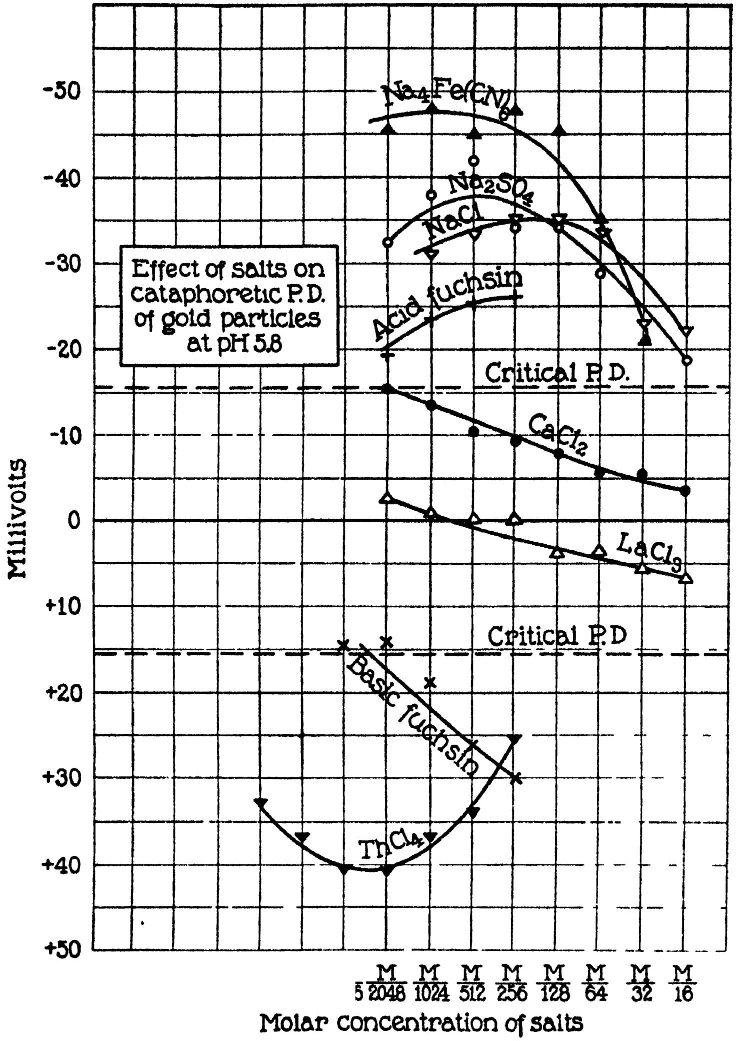

FIG. 4. Influence of salts on the cataphoretic P.D. of particles of gold near neutrality (pH 5.8). Only the effect of concentrations of salts of M/2,048 or above are given.

II.

Experiments on Negatively Charged Particles.

Six types of particles, negatively charged at pH 5.8, were selected for this study, collodion (Fig. 1), mastic (Fig. 2), Acheson's graphite

(Fig. 3), gold (Fig. 4), Na gelatinate (Fig. 5), and crystals of tyrosine. (At pH 5.8 the tyrosine is little if at all dissociated.) In Figs. 1 to 5 the abscissæ are the molar concentrations of the salts while the

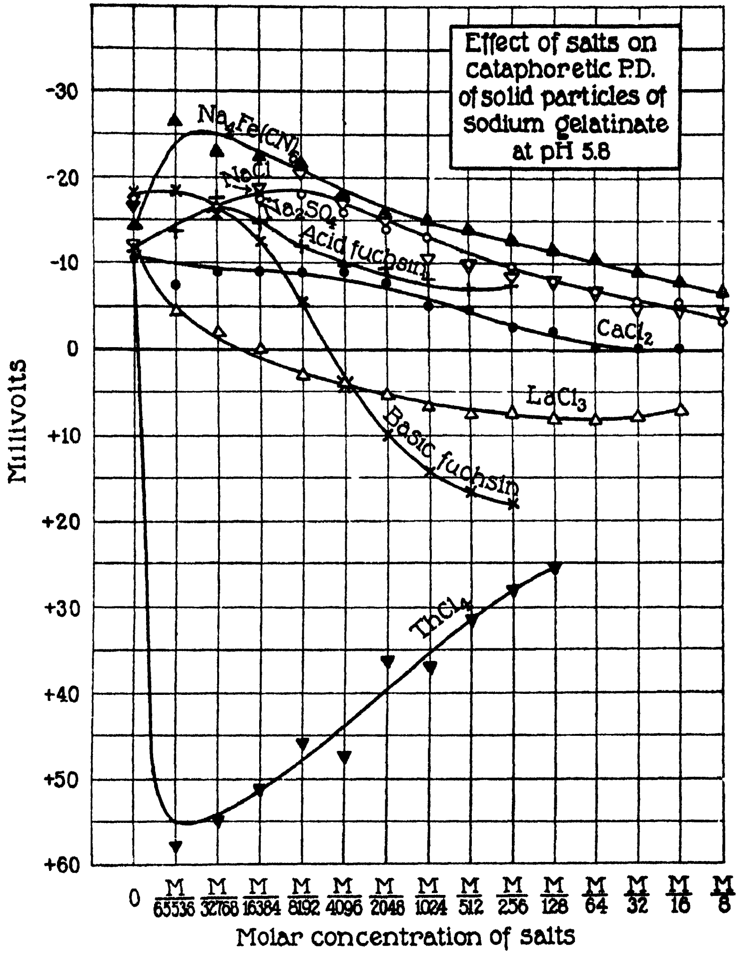

FIG. 5. Influence of salts on the cataphoretic P.D. of solid particles of Na gelatinate near neutrality, pH 5.8.

ordinates are the cataphoretic P.D. in millivolts as calculated from the rate of migration with the aid of the Helmholtz-Perrin formula.[5]

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[5] Freundlich, H., Kapillarchemie, Leipsic, 2nd edition, 1922, 326 ff. Burton, E. F., The physical properties of colloidal solutions, London, New York, Bombay, Calcutta, and Madras, 2nd edition, 1921, 136 ff.

The sign of the charge is always that of the particle the negative charges being above the zero line, the positive charges below. In spite of the wide divergence in the chemical nature of the material, the P.D. curves are in all five figures so similar that the following general rules concerning the influence of salts on the P.D. of negative colloids in distilled water of pH 5.8 hold for all of them. For the purpose of discussion, the curves for the collodion particles will be used (Fig. 1). (In expressing these rules, it is more convenient to speak in terms of the charge of the particles instead of the value of the P.D., which is the quantity actually determined.)

In water near the point of neutrality (*i.e.*, at pH 5.8) salts with monovalent cation raise the negative charge of negative colloids with increasing concentration of the salt until a maximum P.D. is reached; this maximum, which never seems to exceed about 70 millivolts,[6] is the higher the higher the valency of the anion, being a little higher for the $Fe(CN)_6$ than for the Cl ion (see Figs. 1, 2, or 3). What is, perhaps, of theoretically greater importance is the fact that the curve for $Na_4Fe(CN)_6$ rises more steeply than that for either Na_2SO_4 or NaCl. When the characteristic maximal P.D. for each salt is reached, a further addition of the same salt causes a fall of the value of the P.D.

Salts of the type of $CaCl_2$, *i.e.*, with bivalent cation and monovalent anion, also raise the negative charge of the particles as long as the concentration is below M/2,048. This is best seen in Fig. 1, representing the effect of the salts on collodion particles. The original P.D. without salt was only about 7 millivolts (in the former papers a P.D. of about 30 millivolts was found in this case, but this was chiefly due to impurities and other imperfections of the technique which have since been remedied). The maximal P.D. in $CaCl_2$ for collodion particles is, however, only about 28 millivolts as against about 53 for NaCl (Fig. 1).

Even a salt like $LaCl_3$ still raises the P.D. (Fig. 1), but only from 7 to about 15 millivolts. The maximal P.D. is reached in $LaCl_3$ at a molar concentration of between M/16,000 and M/8,000. With the addition of more $LaCl_3$ the P.D. is depressed. At a concentration of

[6] von Hevesy, G., *Kolloid Z.*, 1917, xxi, 129. Lorenz, R., Raumerfüllung und Ionenbeweglichkeit, Leipsic, 1922, 237.

about M/128 the particles become slightly positive, but the P.D. remains at nearly zero even in M/16 $LaCl_3$. In the writer's former experiments the fact that $LaCl_3$ is able to raise the P.D. of collodion particles in very low concentrations was concealed on account of the impurities which had raised the P.D., without the additions of any salt.

No rise occurs, however, in the case of a salt like $ThCl_4$. Extremely low concentrations of this salt make the particles positive, and the P.D. increases with the increase of the concentration, until a maximal P.D. of 55 millivolts is reached at a concentration of $ThCl_4$ between M/30,000 and M/8,000. A further increase in the concentration of the salt depresses the P.D. again (Fig. 1).

TABLE I.

Maximal P.D. *Values in Solutions of* $Na_4Fe(CN)_6$ *at pH 5.8 for Negative Colloids.*

Nature of particles.	P D
	millivolts
Mastic	about 75
Collodion	" 62
Acheson's graphite	" 43
Gold	" 47
Crystals of tyrosine	" 45
Na gelatinate	" 25

High positive charges are also produced by basic fuchsin and the P.D. increases with the concentration of the dye until a maximum is reached after which the P.D. drops with further increase in the concentration of the dye.

If we compare the difference in the relative effect of salts on the six negative colloids (Table I) we may use the maximal P.D. values in $Na_4Fe(CN)_6$ as a standard of comparison.

The values for Na_2SO_4 and NaCl vary correspondingly.

III.

Positively Charged Particles.

Figs. 6, 7, and 8 show the effects of salts on positively charged particles; namely, ferric hydroxide, calcium oxalate, and casein chloride. The experiments with ferric hydroxide and calcium oxalate were made at pH 5.8 and care was taken that this pH was not altered by the addition of the salts (with the exception of the dyes). The experiments with casein chloride had to be made on the acid side of the isoelectric point of casein and the pH of 4.0 was selected. The reader will notice that all three positively charged particles are electrolytes while in the case of the negatively charged particles some, *e.g.*, Na gelatinate, were electrolytes, while collodion and mastic were apparently non-electrolytes.

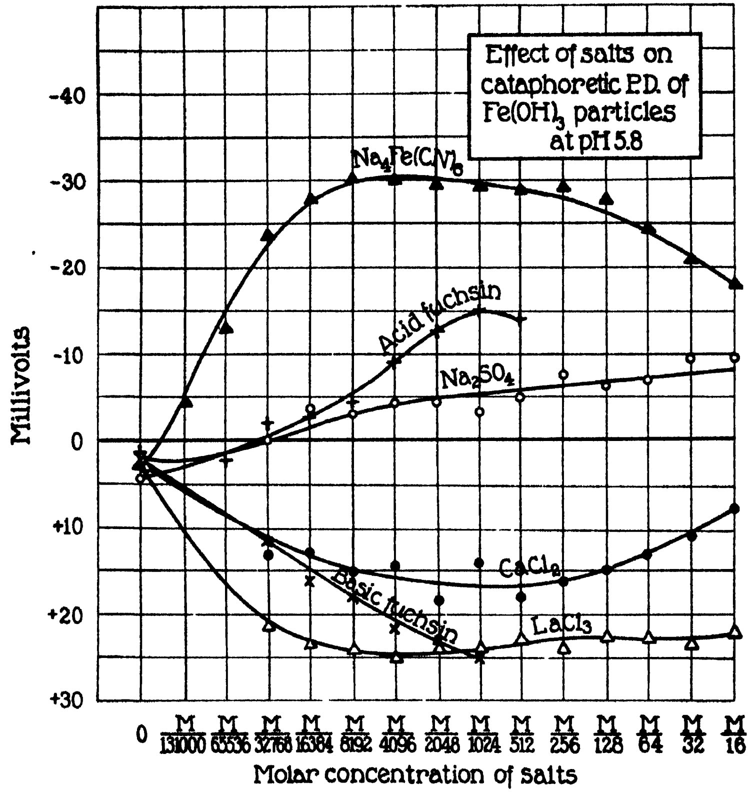

FIG. 6. Influence of salts on the cataphoretic P.D. of particles of $Fe(OH)_3$ near neutrality, pH 5.8.

The curves for the positive colloids differ in an essential respect from those for negative colloids. While in the case of negative colloids the Cl and SO_4 ions had a powerful augmenting effect on the P.D.

these two anions have almost no effect on the positive colloids. Na_2SO_4 makes the positive colloids slightly negative at comparatively high concentrations but the P.D. is very small indeed. Only $Na_4Fe(CN)_6$ and acid dyes make the positive particles negative in rather low molar concentrations, but even here the P.D. remains low (30 millivolts or less).

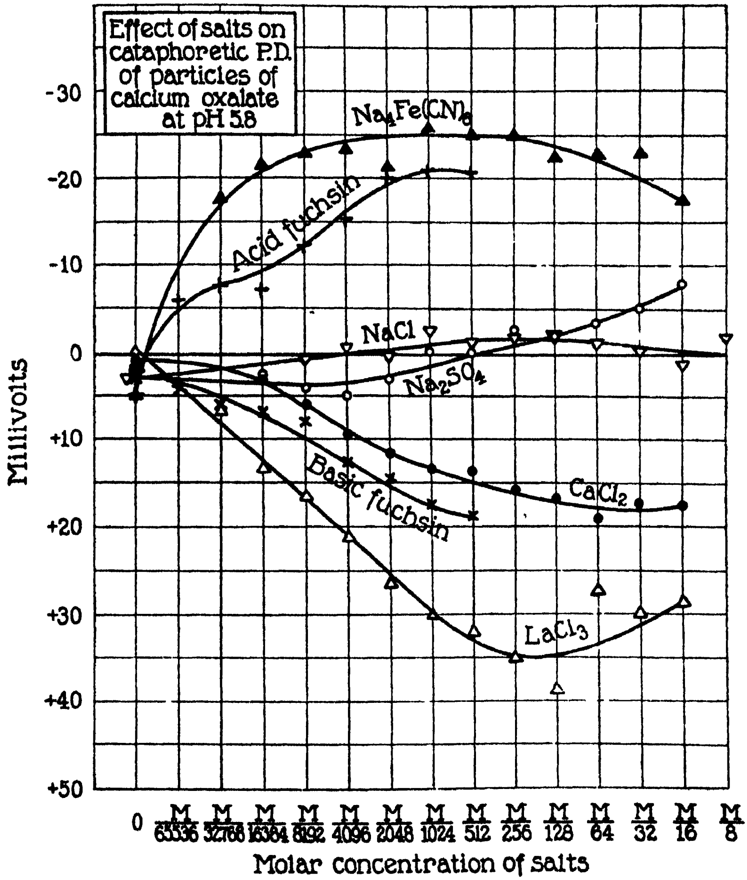

FIG. 7. Influence of salts on the cataphoretic P.D. of particles of calcium oxalate near neutrality, pH 5.8.

Perhaps, it is on account of this lack of efficiency of the Cl ion, that $CaCl_2$ and $LaCl_3$ in low concentrations increase the positive charge of the positive colloids until a maximal P.D. is reached after which a further increase in the concentrations of $CaCl_2$ and $LaCl_3$ will depress the P.D. again.

Basic fuchin dose not act quite as strongly as dose $LaCl_3$ on the P.D. of positive colloids but in the same sense.

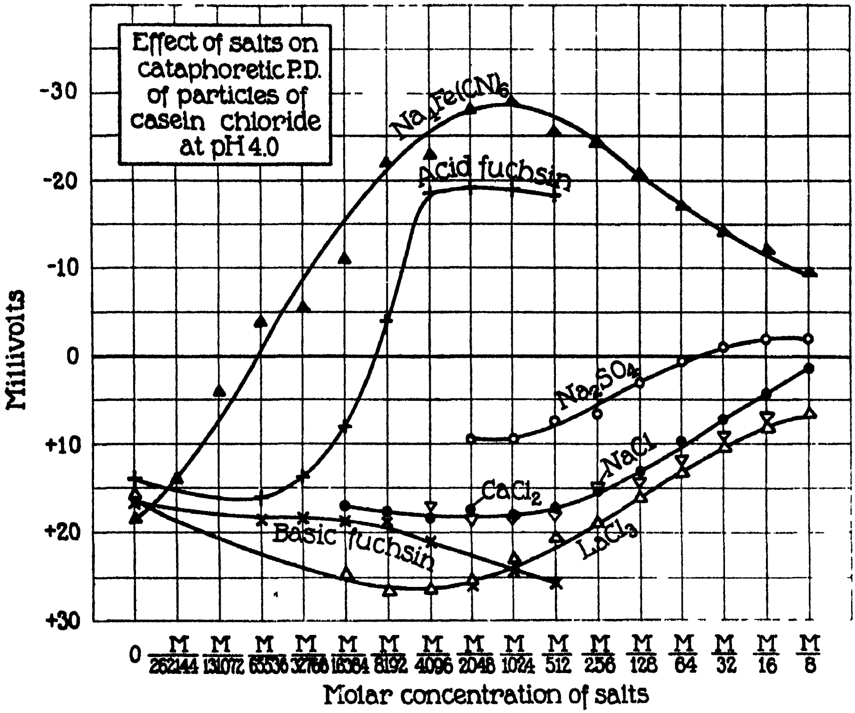

FIG. 8. Influence of salts on the cataphoretic P.D. of particles of casein chloride at pH 4.0.

IV.

The Action of Alkalies and Acids on the Cataphoretic P.D.

The influence of NaOH on the cataphoretic P.D. of different colloidal particles is almost identical with that of $Na_4Fe(CN)_6$. Regardless of whether the colloid is originally negative or positive, NaOH makes the particles always more negative and the P.D. increases until a maximum is reached at about M/4,096 or M/1,024; after which the P.D. drops again upon further increase in the concentration of the alkali (Fig. 9). The OH ion is therefore driven with about the same relative force into the enveloping film as is the Fe(CN)$_6$ ion. Table II gives the maximal values of the P.D. in NaOH solutions.

It might be inferred *a priori* that hydrogen ions act like thorium ions and this would seem to receive support in the statement so often found in the colloidal literature that hydrogen ions make colloids positive. While this is true for amphoteric electrolytes, Fig. 10 shows that this cannot be generalized. Low concentrations of HCl

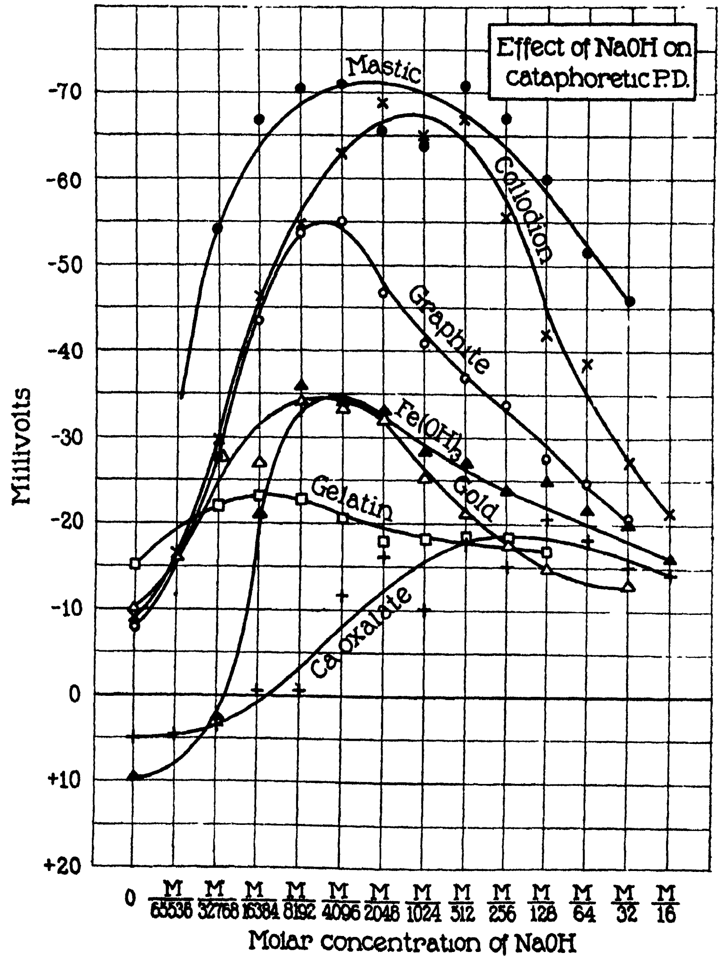

FIG. 9. Influence of NaOH on the cataphoretic P.D. of different kinds of particles.

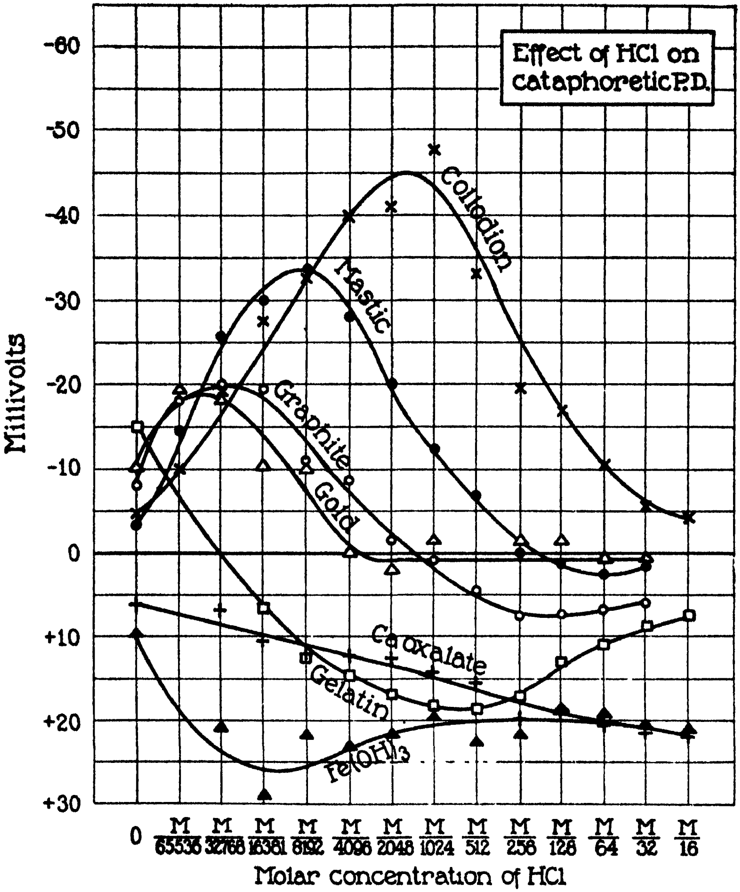

FIG. 10. Influence of HCl on the cataphoretic P.D. of different kinds of particles.

TABLE II.

Maximal P.D. *between Different Particles and Solutions of NaOH.*

Nature of particles.	P D.
	millivolts
Mastic	71
Collodion	67
Acheson's graphite	55
Gold	50
$Fe(OH)_3$	35
Na gelatinate	23
Ca oxalate	18

generally increase the negative charge of negative colloids such as collodion, mastic, Acheson's graphite, and gold (Fig. 10) until a maximum is reached, which is quite high in the case of collodion (about 45 millivolts), lower in the case of mastic (about 33 millivolts), graphite, and gold (about 20 millivolts). A further increase in the concentration of the HCl depresses the P.D. (Fig. 10).

In the case of collodion the sign of charge of the particles is not reversed by acid, the collodion particles remaining negative even in M/8 solutions of HCl (Fig. 10). Particles of mastic or of gold may become positive in higher concentrations of HCl, but the P.D. remains very low (2 or 3 millivolts). Graphite becomes slightly more positive in acid, but the P.D. is also low (about 7 millivolts). HCl is therefore even a little less active than $LaCl_3$ which also makes negative colloids positive only at comparatively high concentrations and the P.D. is also never high in this case.

The statement that acids render negative colloids strongly positive seems to be correct only in such cases where the chemical character of the colloid is changed by the acid. Thus amphoteric electrolytes like the proteins are transformed by HCl into protein chlorides where the non-diffusible ion of the particle is a positive protein ion. Particles of Na gelatinate are negatively charged (Fig. 4) but if enough HCl is added the protein is transformed into gelatin chloride where the non-diffusible protein ion is a cation. Gelatin chloride particles may reach a considerable positive P.D. (Fig. 10).

McTaggart[7] states that $Th(NO_3)_4$ makes gas bubbles positive and so does also to a slight extent $LaCl_3$; it would be very important to find out whether or not HCl can produce a considerable positive charge on gas bubbles. The experiments of McTaggart do not seem to give a definite answer to this question. It seems, therefore, that for the present we must discriminate in the effects of HCl on the cataphoretic P.D. between cases where the chemical nature of the particles is changed by the acid and where no such changes occur. In cases of the latter type (presumably collodion particles), solutions of

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[7] McTaggart, H. A., *Phil. Mag., Series 6*, 1914, xxvii, 297; xxviii, 367; 1922, xliv, 386.

NaOH and HCl may act like neutral solutions of NaCl on the cataphoretic P.D. as is shown in Fig. 11, the only difference between the three electrolytes being that the OH ion acts a little more strongly than the Cl ion and the H ion slightly more strongly than the Na ion;

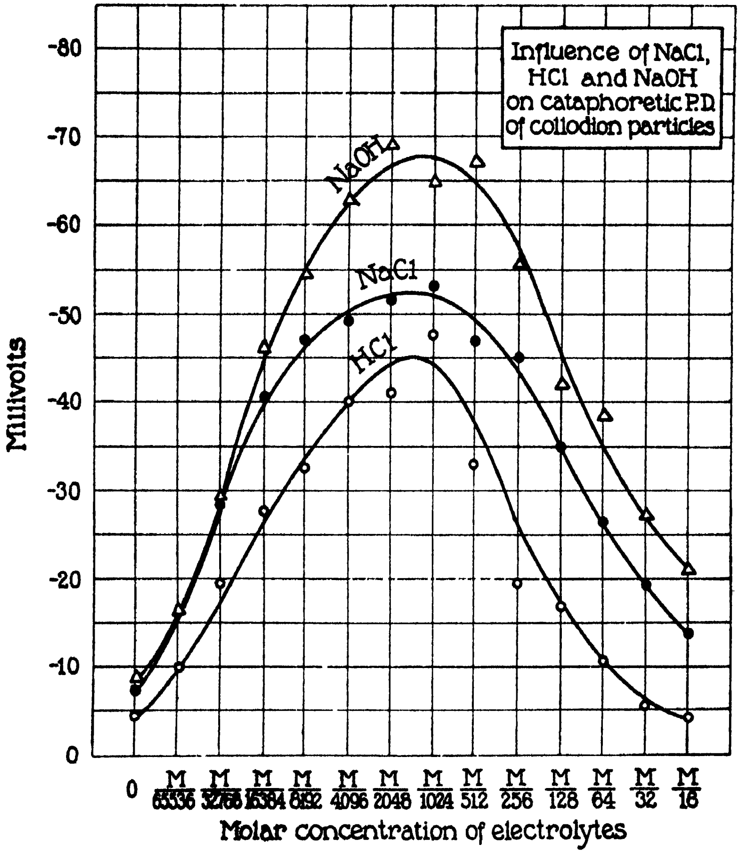

FIG. 11. Comparison of the influence of HCl, NaCl, and NaOH on the cataphoretic P.D. of collodion particles. The curve for NaCl represents the effects of this salt at an approximately neutral reaction, pH 5.8.

or, if we use the suggestion of a relative orientation of the ions, along the normal to the interface, OH ions are driven with a slightly greater force into the enveloping film than the Cl ions, and the H ions are driven with a slightly smaller force away from the interface than the Na ions.

V.

Flocculation.

Although it is not an essential part of our problem it may perhaps be of importance to point out that there seems to exist in the case of all of these suspensions a critical P.D. below which they cease to be stable and commence to flocculate. It is rather remarkable that this critical P.D. differs very little with the chemical nature of the suspended particles. The critical P.D. is indicated by lines marked Critical P.D. in Figs. 2 and 4. The suspensions of collodion,[2] mastic, graphite, and gold cease to be stable and flocculation commences when the cataphoretic P.D. falls to a value of between 16 and 13 millivolts. Northrop and De Kruif[8] found a similar value, namely 15 millivolts, for the flocculation of certain bacteria. The values for the flocculation of suspensions of calcium oxalate, denatured casein, and denatured albumin[9] are not very far from this value. The interpretation of this fact seems to be that flocculation can only occur when the average velocity of the particles is high enough to overcome the repelling force due to the P.D. of the double layer. The average velocity of the particles seems to be such that it suffices to overcome the repulsion due to a P.D. of about 14 millivolts or less. Flocculation requires, however, something more than the mere overcoming of the repulsive forces due to the cataphoretic P.D.; namely, the force of cohesion between the particles themselves must be considerably greater than the forces of adhesion between the particles and the water. Particles of gelatin (or of collodion coated with gelatin) will not flocculate even if the P.D. is zero, and the same is true for particles of denatured egg albumin or casein in the presence of gram molecular concentrations of NaCl or $CaCl_2$.[9] In these cases the forces of cohesion of the particles are relatively small compared with the attraction of the particles for water, so that they fail to cohere upon collision even if the cataphoretic P.D. is zero.

It has frequently been stated that the precipitating power of electrolytes increases with the mobility of the active ion.[10] Thus the precipitating power of the chlorides of alkali metals is said to increase in the order Li < Na < K < Rb < Cs. This cannot be a general rule, since LiCl, NaCl, and KCl influence the P.D. of collodion as well as graphite particles in an identical way. Flocculation is determined by the critical P.D. and since identical concentrations of LiCl, NaCl, and KCl are required to bring about the critical P.D., the flocculating concentrations for these salts should be identical. This was found to be true for suspensions of mastic, graphite, and collodion when care was taken to keep the pH constant. In Tables III, IV, and V are given the flocculating concentrations of different salts at pH 5.8 for suspensions of mastic, Acheson's graphite, and gold. (Only in the case of $ThCl_4$ and basic fuchsin were the pH different.) By flocculation is meant the complete settling of the particles, leaving a completely colorless and clear supernatant liquid; while by suspension is meant that the liquid remains red in the case of gold or gray or opaque in the case of mastic or black in the case of graphite; while no or little settling occurs. The second column of the tables gives the minimal molar concentration required for flocculation, and the third column the highest concentration at which the suspensions remain stable for weeks. In order to get unequivocal results, the values in Columns

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[8] Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 639.

[9] Loeb, J., *J. Gen. Physiol.*, 1922–23, v, 479.

[10] Mukherjee, J. N., *Farad. Soc. Rep.*, London, 1921, xx, 103.

TABLE III.

Mastic.

	Flocculation.	Suspension.
LiCl	M/4	M/8
NaCl	M/4	M/8
KCl	M/4	M/8
RbCl	M/2	M/4
CsCl	M/4	M/8
$MgCl_2$	M/32	M/64
$CaCl_2$	M/32	M/64
$SrCl_2$	M/32	M/64
$BaCl_2$	M/32	M/64
$MnCl_2$	M/32	M/64
$LaCl_3$	M/8,192	M/16,000
$ThCl_4$	M/260,000	$\lessgtr$ M/260,000
Basic fuchsin	M/8,000 – M/4,000	> M/4,000 < M/8,000

TABLE IV.

Graphite (Aquadag).

	Flocculation.	Suspension.
LiCl	M/32	M/64
NaCl	M/32	M/64
KCl	M/32	M/64
RbCl	M/32	M/64
CsCl	M/32	M/64
$MgCl_2$	M/512	M/1,024
$CaCl_2$	M/1,024	M/2,048
$SrCl_2$	M/512	M/1,024
$BaCl_2$	M/1,024	M/2,048
$MnCl_2$	M/1,024	M/2,048
$LaCl_3$	M/65,000	M/130,000
$ThCl_4$	M/500,000	≶ M/500,000
Na_2SO_4	M/64	M/128
$Na_4Fe(CN)_6$	M/64	M/256
$MgSO_4$	M/512	M/1,024
Basic fuchsin	M/8,000 and M/16,000	>M/8,000 <M/16,000
HCl	M/2,048	M/4,096

TABLE V.

Gold.

	Flocculation.	Suspension.
LiCl	M/16	M/32
NaCl	M/64	M/128
KCl	M/128	M/256
RbCl	M/16	M/32
CsCl	M/128	M/256
$MgCl_2$	M/16,000	M/32,000
$CaCl_2$	M/4,096	M/8,192
$SrCl_2$	M/8,192	M/16,000
$BaCl_2$	M/16,000	M/32,000
$MnCl_2$	M/8,192	M/16,000
$LaCl_3$	M/260,000	M/500,000
$ThCl_4$	M/500,000	
Na_2SO_4	M/64	M/128

2 and 3 were ascertained after the suspensions had been standing in test-tubes for a week or more at room temperature. Variations occurred only in the case of gold, but they were too irregular and could not be reproduced with any degree of certainty. The writer cannot suppress a suspicion that the variations in the flocculating concentrations of salts in the case of gold particles were the result of traces of impurities introduced accidentally. In Table V only the red suspensions were permanently stable, while those which at the beginning were blue or bluish red had settled.

Odén[11] observed enormous differences in the precipitating power of the salts of the alkali metals on suspensions of colloidal sulfur. Nothing of this kind can be observed in the case of the precipitation of mastic, Acheson's graphite, collodion particles, or even particles of gold.

VI.

Theoretical Remarks.

There are two kinds of forces which can cause an excess of one kind of ions in the enveloping film; namely, forces inherent in the water itself and forces of attraction between the colloidal particles and the ions.

The share which forces inherent in the water have in the orientation of the oppositely charged ions of an electrolyte in the interface can only be ascertained through experiments on the influence of electrolytes on the cataphoretic migration of gas bubbles in aqueous solutions, but experiments of this kind are as yet too incomplete to permit more than the statement that in cataphoresis gas bubbles are generally negatively charged, that their sign of charge is reversed by low concentrations of $Th(NO_3)_4$ and presumably by higher concentrations of $LaCl_3$; and that it is doubtful whether acids alone can cause a noticeable reversal in the sign of charge of gas bubbles (*i.e.* with a P.D. exceeding a few millivolts). If we apply these meager data to the experiments reported in this paper we may say that the effect of $ThCl_4$ or $LaCl_3$ or even of strong acids on dielectrics like collodion or mastic are similar to those observed on gas bubbles and that therefore the effects of these three groups of electrolytes on the cataphoretic P.D. of dielectrics may be partly or entirely due to forces inherent in the water itself. Correspondingly it is quite possible that the effect of $Na_4Fe(CN)_6$ which reverses the sign of charge of positive particles may turn out to be due to forces inherent in the water itself. But we cannot yet state with any certainty that the same is true for the effects of ions of lower valency on the cataphoretic P.D. of solid particles.

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[11] Odén, S., Der kolloide Schwefel, *Nova acta regiæ Soc. Sc. Upsaliensis, Series 4*, 1913, iii, No. 4.

We can say, however, that where the effects of an electrolyte on the cataphoretic P.D. differ with the nature of the solid particles the forces or conditions inherent in the solid particles must be responsible. The effects of this type of forces and of the forces inherent in the water may be superposed upon each other.

That some particles assume a positive charge in water in the absence of electrolytes can only be due to the chemical nature of the particle, whereby cations (including the hydrogen ions of the water) are more strongly attracted by the particle than the anions. It is noteworthy that the positive colloids mentioned in this paper are all electrolytes. Negative colloids may be electrolytes or non-electrolytes, but since gas bubbles are generally negatively charged, the charge of negative colloids need not be entirely due to a preferential attraction of anions by the solid particle but may be due partly or entirely to the forces inherent in the water as discussed in the preceding papers.[2]

It must be ascribed to forces inherent in the particle that the point of reversal of the sign of charge in the case of proteins coincides with the isoelectric point of the particle.

The considerable variation in the maximal cataphoretic P.D. with the chemical nature of the particle in solutions of $Na_4Fe(CN)_6$ and NaOH (as shown in Tables I and II) must also be ascribed to the influence of the particles themselves.

SUMMARY.

1. The effect of eight salts, NaCl, Na_2SO_4, $Na_4Fe(CN)_6$, $CaCl_2$, $LaCl_3$, $ThCl_4$, and basic and acid fuchsin on the cataphoretic P.D. between solid particles and aqueous solutions was measured near the

point of neutrality of water (pH 5.8). It was found that without the addition of electrolyte the cataphoretic P.D. between particles and water is very minute near the point of neutrality (pH 5.8), often less than 10 millivolts, if care is taken that the solutions are free from impurities. Particles which in the absence of salts have a positive charge in water near the point of neutrality (pH 5.8) are termed positive colloids and particles which have a negative charge under these conditions are termed negative colloids.

2. If care is taken that the addition of the salt does not change the hydrogen ion concentration of the solution (which in these experiments was generally pH 5.8) it can be said in general, that as long as the concentration of salts is not too high, the anions of the salt have the tendency to make the particles more negative (or less positive) and that cations have the opposite effect; and that both effects increase with the increasing valency of the ions. As soon as a maximal P.D. is reached, which varies for each salt and for each type of particles, a further addition of salt depresses the P.D. again. Aside from this general tendency the effects of salts on the P.D. are typically different for positive and negative colloids.

3. Negative colloids (collodion, mastic, Acheson's graphite, gold, and metal proteinates) are rendered more negative by low concentrations of salts with monovalent cation (*e.g.* Na) the higher the valency of the anion, though the difference in the maximal P.D. is slight for the monovalent Cl and the tetravalent $Fe(CN)_6$ ions. Low concentrations of $CaCl_2$ also make negative colloids more negative but the maximal P.D. is less than for NaCl; even $LaCl_3$ increases the P.D. of negative particles slightly in low concentrations. $ThCl_4$ and basic fuchsin, however, seem to make the negative particles positive even in very low concentrations.

4. Positive colloids (ferric hydroxide, calcium oxalate, casein chloride—the latter at pH 4.0) are practically not affected by NaCl, are rendered slightly negative by high concentrations of Na_2SO_4, and are rendered more negative by $Na_4Fe(CN)_6$ and acid dyes. Low concentrations of $CaCl_2$ and $LaCl_3$ increase the positive charge of the particles until a maximum is reached after which the addition of more salt depresses the P.D. again.

5. It is shown that alkalies (NaOH) act on the cataphoretic P.D. of both negative and positive particles as $Na_4Fe(CN)_6$ does at the point of neutrality.

6. Low concentrations of HCl raise the cataphoretic P.D. of particles of collodion, mastic, graphite, and gold until a maximum is reached, after which the P.D. is depressed by a further increase in the concentration of the acid. No reversal in the sign of charge of the particle occurs in the case of collodion, while if a reversal occurs in the case of mastic, gold, and graphite, the P.D. is never more than a few millivolts. When HCl changes the chemical nature of the colloid, *e.g.* when HCl is added to particles of amphoteric electrolytes like sodium gelatinate, a marked reversal will occur, on account of the transformation of the metal proteinate into a protein-acid salt.

7. A real reversal in the sign of charge of positive particles occurs, however, at neutrality if $Na_4Fe(CN)_6$ or an acid dye is added; and in the case of negative colloids when low concentrations of basic dyes or minute traces of $ThCl_4$ are added.

8. Flocculation of the suspensions by salts occurs when the cataphoretic P.D. reaches a critical value which is about 14 millivolts for particles of graphite, gold, or mastic or denatured egg albumin; while for collodion particles it was about 16 millivolts. A critical P.D. of about 15 millivolts was also observed by Northrop and De Kruif for the flocculation of certain bacteria.

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THE LUNG VOLUME IN HEART DISEASE.

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INTRODUCTION.

In heart failure it is assumed that the supply of O_2 to the tissues and the removal of CO_2 from them is not commensurate with the needs of the organism. Pearce (1) has emphasized the intimate relationship existing between respiration, circulation, and metabolism. No longer can any of these functions be regarded as isolated, but they must be considered together as a physiological system. The reason for studying lung volume in heart disease is that it aids in an understanding of the interdependence of cardiac and respiratory failure.

Bohr (2) and Hasselbalch (3) have emphasized the importance of the simultaneous determination of the total lung volume and its subdivisions. In an attempt to visualize the degree of pulmonary distension and the relations between air content in the lung and blood flow in the lesser circulation a knowledge of the vital capacity alone may lead to error. The study of the vital capacity of the lungs in heart disease has become of great practical interest, owing largely to the work of Peabody and his associates (4). Study of the vital capacity alone, however, can throw little further light on the pathologic changes, both structural and functional, which give rise to the phenomenon of dyspnea.

Since Bohr reawakened interest in pulmometry, numerous investigators (Rubow (5), Bruns (6), Bittorf and Forschbach (7), and Siebeck (8)) have studied the total air capacity of the lungs (vital capacity and residual air) in cases of heart disease. These investigators used the gas mixture method described by Bohr (2) for the determination of the volume of residual air. Two of these investigators (Bruns (6) andSiebeck(8)), dwell with considerable emphasis on the difficulty of applying Bohr's method to patients with heart disease in whose lungs normal gaseous diffusion, they believe, is disturbed. In this country, Peters (9), using the method of Lundsgaard and Van Slyke (10), found the decrease in vital capacity in heart disease to be accompanied by a decrease in total lung capacity and residual air. Lundsgaard's (11) recent important contributions to the subject will be discussed later. In our hands the procedure of mixing used by Lundsgaard and Van Slyke (10) has not proved applicable to the study of those patients with heart disease in whom any considerable degree of dyspnea is present. This fact has been discussed in another communication (12) in which a new method for measuring lung volume was described. The present paper deals with the results obtained by applying this method in the study of a series of cases of heart disease.

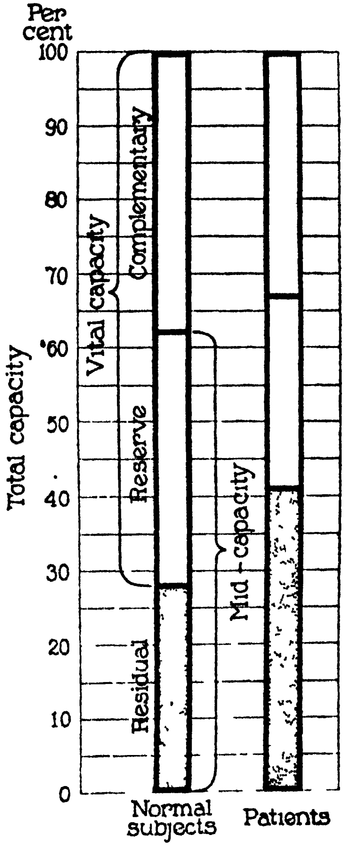
Material.—The material for study was selected from a group of cases suffering from various types of chronic heart disease.

Most of the patients had disturbances of the cardiac mechanism as evidenced by the presence of auricular fibrillation. The advantage of studying patients with fibrillation of the auricles lies in the fact that the heart rate and usually the state of decompensation in these patients can be controlled by the administration of digitalis. It was our practise in most cases to put the patient to bed on admission and to administer digitalis, usually in the form of digitan, Merck. When the patient's cardiorespiratory system had reached a more or less stable condition, lung volume determinations were made. The subject could at this time be introduced to the use of the method under the most propitious circumstances. The patient was then studied during various stages of decompensation and compensation. All lung volumes were determined with the patient sitting upright in a wheel chair. The patients were brought from the wards to the laboratory, where they were allowed to sit quietly for about ½ hour before observations were begun. In as far as possible an effort was made to exclude cases which gave evidence of suffering from bronchopneumonia, bronchitis, asthma, or emphysema, so that the changes described may be attributed in the main to circulatory failure and its concomitant phenomena. One case of the series had a considerable transudation of fluid into the pleural cavities. This case was studied before and after thoracentesis and aspiration of the fluid.

A number of normal individuals of both sexes were also studied under circumstances similar to those existing during the study of the patients.

Definition of Terms.—The terminology used in this paper is that of Panum (13) and Bohr (2), which has been reviewed by Lundsgaard and Van Slyke (10).

1. Mid-position = point half way between normal expiration and normal inspiration.
2. Complementary air = air expired from maximum inflation to mid-position.
3. Reserve air = air expired from mid-position to maximum deflation.
4. Vital capacity = air expired from maximum inflation to maximum deflation.
5. Residual air = air remaining in lungs after fullest expiration. This includes the so called dead air space.
6. Middle capacity = total air held in lungs at mid-position.
7. Total capacity = total air held in fully inflated lungs.



TEXT-FIG. 1. The total lung volume is represented as 100. The two columns show a comparison of normal subjects and patients with respect to the average percentage of the total occupied by its subdivisions.

The diagram shown in Text-fig. 1 will make our use of the terms clear. It is to be emphasized that this diagram does not depict the lung volumes from a functional or physiological point of view, but simply shows the relative volumes of the various subdivisions of the total lung capacity.

Relative Lung Volumes, or Subdivisions of Total Lung Volume, Calculated as Percentages of Total Lung Volume.

If the volume of each subdivision of total lung volume is expressed as a percentage of the total lung volume a striking constancy in the figures is found for normal individuals. Table I presents these values as observed in seven normal men and seven normal women.

TABLE I.

Name.	Sex.	Vital capacity. / Total capacity	Residual air. / Total capacity	Middle capacity. / Total capacity	Reserve air. / Total capacity	Complementary air. / Total capacity.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
A.	M.	69	31	63	32	37
H.	"	79	21	52	31	48
E.	"	79	21	53	33	47
B.	"	76	24	55	31	45
D.	"	71	29	69	40	31
Br.	"	72	28	66	38	26
Pl.	"	72	28	63	35	37
Mean value.		74±3	26±3	60±6	34±3	39±7
J.	F.	74	26	65	40	35
C.	"	63	38	68	31	32
Ho.	"	73	27	55	29	44
N.	"	63	37	67	28	35
R.	"	69	31	65	34	35
S.	"	73	27	64	34	36
H.	"	71	29	63	34	37
Mean value.		69±4	31±4	64±3	33±3	36±2

The mean values with average deviations as observed in these fourteen normal subjects including both sexes is shown in Table II.

These values agree closely with those of other investigators (Bohr (2), Rubow (5), Lundsgaard and Van Slyke (10), and Lundsgaard

TABLE II.

$\frac{\text{Vital capacity}}{\text{Total capacity}}$.	$\frac{\text{Residual air}}{\text{Total capacity}}$.	$\frac{\text{Middle capacity}}{\text{Total capacity}}$.	$\frac{\text{Reserve air}}{\text{Total capacity}}$.	$\frac{\text{Complementary air}}{\text{Total capacity}}$.
per cent	*per cent*	*per cent*	*per cent*	*per cent*
72 ± 4	28 ± 4	62 ± 4	34 ± 3	38 ± 6

TABLE III.

Case No.	Sex.	$\frac{\text{Vital capacity}}{\text{Total capacity}}$	$\frac{\text{Residual air}}{\text{Total capacity}}$.	$\frac{\text{Middle capacity}}{\text{Total capacity}}$.	$\frac{\text{Reserve air}}{\text{Total capacity}}$.	$\frac{\text{Complementary air}}{\text{Total capacity}}$.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
1	M.	47	53	80	20	27
		50	50	68	18	32
		62	38	62	23	39
		70	30	62	32	38
2	"	53	47	65	18	35
		57	43	65	22	35
		61	39	60	22	40
3	"	50	50	70	20	30
		61	39	66	26	34
		64	36	67	31	33
4	"	58	42	57	16	43
		63	37	57	19	44
Mean value.		58	42	65	22	36
5	F.	60	40	73	33	27
6	"	62	38	67	29	33
7	"	63	37	66	29	34
8	"	66	34	65	32	35
9	"	50	50	69	19	31
10	"	62	38	67	29	33
		64	36	68	32	32
11	"	50	50	71	21	29
Mean value.		60	40	68	28	32

Where several determinations are given for the same patient they were made during progressive improvement from the decompensated toward the compensated state. In making the averages each observation is counted as a separate case, because of the varying physiological state of the patient.

(11)). Lundsgaard (11) published a similar table prepared from figures taken from the literature and from his own work. Practically all the figures fall within the limits set by the average deviations of Table II.

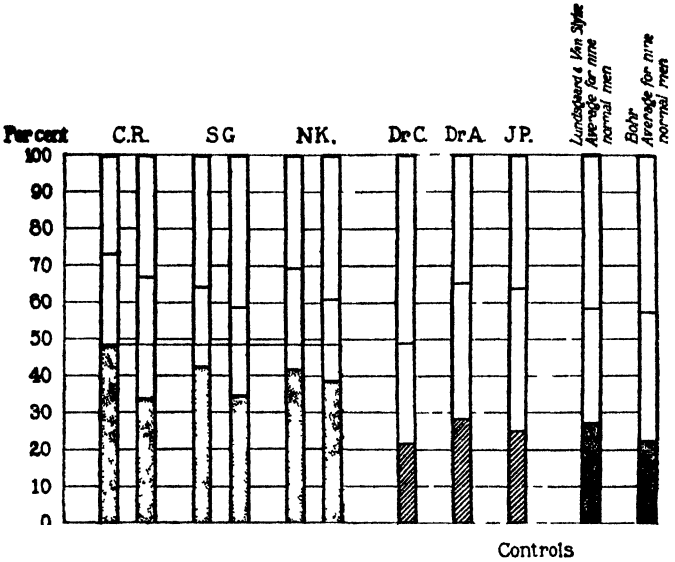
These two tables should be compared with Tables III and IV, in which the lung volumes of a group of patients with valvular heart disease showing evidence of respiratory insufficiency (dyspnea on slight exertion) are presented.

Inspection of Table III shows that as compensation progresses there occurs an increase in the relative vital capacity and a reciprocal decrease in residual air. In most instances the relative mid-capacities decrease and the percentage of reserve air and complementary air to total volume increases. Table IV shows the averages of the relative lung volumes determined in twenty observations on eleven subjects with heart disease, compared with the averages of similar determinations in fourteen observations on fourteen normal individuals. The differences between the normal and pathological lung volumes are well brought out, and it will be observed that changes concomitant with compensation are in the direction of restoration toward normal relative lung volumes.

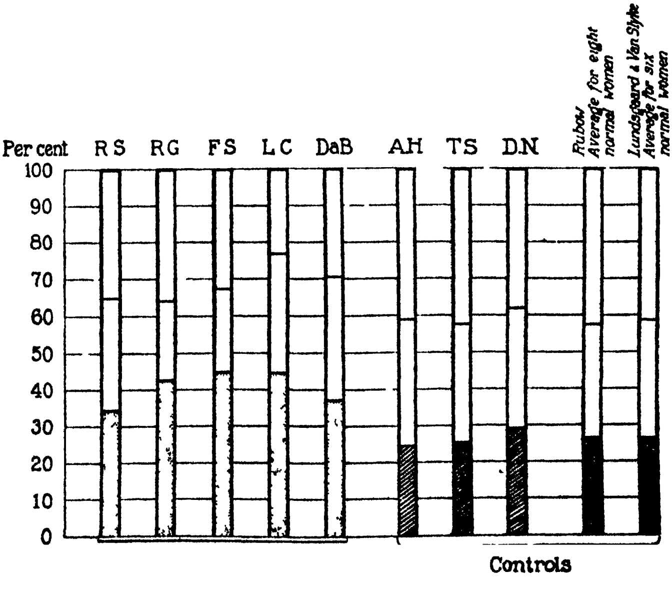
TABLE IV.

Individuals.	$\frac{\text{Vital capacity}}{\text{Total capacity}}$.	$\frac{\text{Residual air}}{\text{Total capacity}}$.	$\frac{\text{Middle capacity}}{\text{Total capacity}}$.	$\frac{\text{Reserve air}}{\text{Total capacity}}$.	$\frac{\text{Complementary air}}{\text{Total capacity}}$.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Patients.	59	41	67	25	34
Normals.	72 ± 4	28 ± 4	62 ± 4	34 ± 3	38 ± 6

These averages, presented graphically in Text-figs. 1 to 3, show the relative lung volumes for normal men and women as compared with the relative lung volumes determined by other authors for normal individuals, and with relative lung volumes for patients with heart disease.

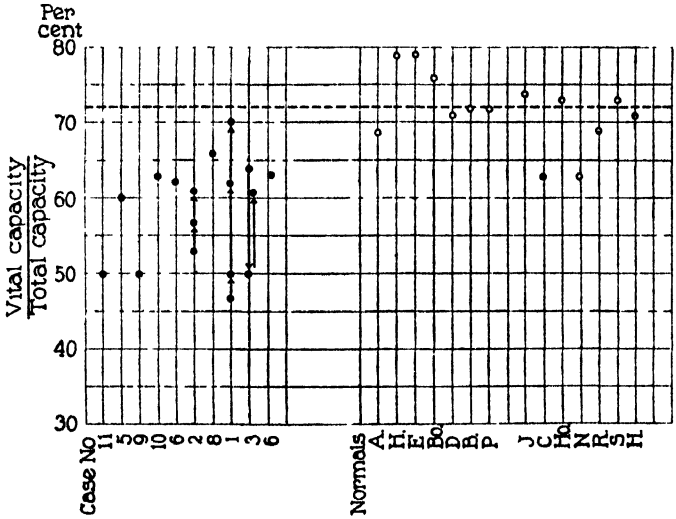


TEXT-FIG. 2. Percentage relation of vital capacity, mid-capacity, and residual air in three male patients compared with the same in normal subjects. In each of the three patients the second column represents the changes following rest and digitalis therapy.



TEXT-FIG. 3. Percentage relation of vital capacity, mid-capacity, and residual air in five female patients compared with the same in normal subjects.

The functional significance of these changes is not clear, though it is evident that any quantitative deviation from a normal relationship is likely to be of importance. *Per se* they show nothing of actual changes in the capacity of the lung. From such a relationship as is shown in Text-fig. 1 it may be concluded that the patient with heart disease is able to use less of his whole lung capacity in forcible ventilation than is the normal subject. In spite of the relative increase in mid-capacity it is apparent from Text-fig. 1 that the cardiac patients are breathing with lungs which are nearer the position of complete expiration (diminished reserve), though this position may itself be elevated (relatively increased residual air). The pronounced increase in the percentage relation of vital capacity to total capacity during compensation has suggested that this value might be used as a quantitative expression of the state of compensation. This in the main is true, as shown in Text-fig. 4, in which the $\frac{\text{Vital capacity}}{\text{Total capacity}}$ per cent is graphically presented. It is seen that an increase during compen-



TEXT-FIG. 4. Percentage relation of vital capacity to total capacity in ten patients compared to the same in fourteen normal individuals. The arrows pointing upward indicate changes in an individual patient from the decompensated to the compensated state. Arrows downward indicate the reverse change.

sation and decrease during decompensation occur. The fact that the level of the values for patients with heart disease is lower than the values for normal individuals is also indicated. In the more severely decompensated cases in which, as will be shown later, there is a decrease in residual air as well as in vital capacity, the significance of this value is lost, as there is a tendency for it to return to normal.

One of the chief objects in determining relative lung volumes in normal individuals is that with these figures the establishing of normal standards is facilitated. If a standard for any one of the lung volumes for individuals of a given weight or height or chest measurement is established, it will be possible to calculate on a percentage basis the other lung volumes normal for these individuals.

Absolute Lung Volumes[1] *Normal Standards.*

In order to determine whether an individual's lung volumes are larger or smaller than normal, it is obvious that normal standards must be available. In establishing standards of normal vital capacity, the relation of height of the individual to vital capacity has been used by Hutchinson (14) and Peabody (4), the relation of surface area to vital capacity by Peabody (4) and Dreyer (15), and the relation of chest volume to vital capacity by Lundsgaard and Van Slyke (10). West (16) made a valuable comparative study of these various methods for determining normal standards and concluded that the method using the relation of surface area to vital capacity was the most satisfactory.

In applying a surface area (height-weight) formula in the study of pathological conditions one is, of course, confronted with the errors associated with abnormalities in weight due to the disease. An effort was made to avoid this difficulty as far as possible by using the patient's weight only when he was in water balance and when there was no evidence of edema.

In the use of chest volume measurement for calculation of normal lung volumes Lundsgaard recommends that the measurements be made during forced inspiration, assuming that of the various positions occupied by the patient's chest during respiration the position taken during forced inspiration is the nearest to normal. We are not prepared to defend the assumption of Lundsgaard that the forced inspiratory chest position of the patient is the one nearest to the normal, but if the chest volume method is employed, this figure is probably the best one to use at present. As will be seen both surface area and chest volume have been used in the present study in determining standards for calculating normal lung volumes. We cannot decide between the merits of the two methods. To do this would require study of a series of some thousand individuals of all sorts and conditions, such as Hutchinson (14) studied. With either method one encounters occasional normal persons whose observed lung volumes do not agree with the calculated ones.

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[1] Lung volumes are expressed in liters at temperature and pressure at which they were observed. They have not been reduced to 0°C. and 760 mm. Hg.

From the calculated vital capacity the other lung volumes were derived, using the normal relative lung volumes according to the following example.

Height 175 cm.; weight 77 kilos; surface area 1 92 sq. m.

Liters

1.92 × 2.5* = 4 80 (vital capacity).
$\frac{4\ 80}{72}$ = 6.66 (total ").
6 66 − 4.80 = 1 86 (residual air).
6.66 × 34 = 2.26 (reserve ").
6.66 × 38 = 2.53 (complementary air)
6.66 × 62 = 4.13 (mid-capacity).

* 2.5 is West's factor for surface area

These figures were then divided into the observed values and the per cent deviations from normal were thus obtained for the series of normal individuals and for patients with heart disease.

The sources of error in this type of calculation are, of course, very great, and too much importance cannot be attached to conclusions drawn therefrom, except when a uniform deviation from the normals is found in the pathological cases.

The percentage relationship of observed to calculated lung volumes in the normal and pathological series is shown in Tables V and VI, and the mean for each lung volume is graphically presented in Text-fig. 5. Inspection of this figure will show immediately that in the pathological cases the vital capacity and its subdivision (reserve and complementary air) and the mid-capacity and total capacity are diminished, *i.e.* the observed are below the calculated values, whereas in the normal subjects there is close agreement between the observed and calculated values. As regards the residual air, however, the values obtained in the pathological and normal cases agree within the limits of the mean deviations for normals. From this alone one would be led to the conclusion that the volume of the residual air was unchanged in cases of heart disease.

The problem, however, cannot be dismissed so easily and requires further examination. An analysis of the data from which the means shown in Text-fig. 5 were derived is graphically presented in Text-fig. 6, in which each dot represents a separate determination and a comparison of the values in normal and pathological cases can be made by simple inspection. Here it will be seen again that the values obtained in all the observations made on patients for vital capacity, total capacity, and, with two exceptions, mid-capacity fall below the normal line. And it can be safely concluded from this that in patients with heart disease these three lung volumes are usually diminished or at least never increased. The values obtained in determination of residual air fall both above and below the normal line in both series and the scattering of points is so great that no final conclusions can be drawn. The scattering is doubtless in part due to errors in calculating the residual air, since the error involved in this calculation is greater than it is for the larger lung volumes. Among the patients the variations seem to be greater than among the normal individuals, and apparently more patients show an increased residual air than do the normals. This, however, may be accidental and cannot serve as convincing evidence. To clear this point, recourse was had to a study of individual cases in varying stages of decompensation, and a determination of their lung volumes was made and these were compared with the values calculated to be normal for them on the basis of their chest measurements.

Text-figs. 7 to 13 present schematically the absolute lung volumes in liters. The general condition of the patients and state of decompensation may be judged from the curves in the upper portion of the charts and from the symptoms listed below the lung volume columns. The curves represent changes in weight, respiratory rate,

TABLE V.

Comparison of Observed to Calculated Lung Volumes in Fourteen Normal Individuals.

Name.	Sex.	Height.	Weight.	Surface area.	Vital capacity $\left(\frac{\text{Observed}}{\text{Calculated}} = \text{per cent}\right)$.	Total capacity $\left(\frac{\text{Observed}}{\text{Calculated}} = \text{per cent}\right)$.	Residual air $\left(\frac{\text{Observed}}{\text{Calculated}} = \text{per cent}\right)$.	Middle capacity $\left(\frac{\text{Observed}}{\text{Calculated}} = \text{per cent}\right)$.	Reserve air $\left(\frac{\text{Observed}}{\text{Calculated}} = \text{per cent}\right)$.	Complementary air $\left(\frac{\text{Observed}}{\text{Calculated}} = \text{per cent}\right)$.
		cm.	*kg.*	*sq.m.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
A.	M.	163.5	56.1	1.60	$\frac{3.90}{4.00} = 98$	$\frac{5.65}{5.56} = 102$	$\frac{1.75}{1.56} = 112$	$\frac{3.54}{3.45} = 102$	$\frac{1.79}{1.89} = 95$	$\frac{2.11}{2.11} = 100$
Bo.	"	175.3	77.0	1.92	$\frac{5.38}{4.80} = 112$	$\frac{7.11}{6.66} = 107$	$\frac{1.73}{1.86} = 93$	$\frac{3.93}{4.13} = 95$	$\frac{2.20}{2.27} = 97$	$\frac{3.18}{2.53} = 126$
Br.	"	179.2	66.2	1.84	$\frac{4.10}{4.60} = 89$	$\frac{5.73}{6.39} = 90$	$\frac{1.63}{1.79} = 91$	$\frac{3.78}{3.97} = 95$	$\frac{2.15}{2.17} = 99$	$\frac{1.95}{2.43} = 80$
D.	"	178.6	63.0	1.80	$\frac{5.20}{4.50} = 116$	$\frac{7.36}{6.25} = 118$	$\frac{2.16}{1.75} = 123$	$\frac{5.08}{3.88} = 131$	$\frac{2.92}{2.13} = 137$	$\frac{2.28}{2.38} = 96$
H.	"	185.5	67.0	1.92	$\frac{6.10}{5.29} = 115$	$\frac{7.80}{7.35} = 106$	$\frac{1.71}{2.06} = 83$	$\frac{3.86}{4.56} = 85$	$\frac{2.15}{2.50} = 86$	$\frac{3.95}{2.80} = 141$
E.	"	166.8	57.5	1.64	$\frac{4.73}{4.13} = 115$	$\frac{5.99}{5.74} = 104$	$\frac{1.26}{1.61} = 78$	$\frac{3.01}{3.56} = 85$	$\frac{1.75}{1.95} = 90$	$\frac{2.98}{2.18} = 137$
P.	"	180.6	77.4	1.98	$\frac{5.37}{4.95} = 108$	$\frac{7.42}{6.88} = 108$	$\frac{2.05}{1.93} = 106$	$\frac{4.67}{4.27} = 109$	$\frac{2.62}{2.34} = 112$	$\frac{2.75}{2.61} = 105$

N.	F.	153.0	60.1	1.57	$\frac{2.48}{3.14} = 79$	$\frac{3.92}{4.36} = 90$	$\frac{1.44}{1.22} = 118$	$\frac{2.54}{2.70} = 94$	$\frac{1.10}{1.48} = 74$	$\frac{1.38}{1.66} = 83$
R.	"	170.2	70.0	1.82	$\frac{3.81}{3.64} = 105$	$\frac{5.50}{5.06} = 109$	$\frac{1.69}{1.42} = 119$	$\frac{3.57}{3.14} = 114$	$\frac{1.88}{1.72} = 109$	$\frac{1.93}{1.92} = 101$
S.	"	160.9	56.4	1.59	$\frac{2.83}{3.18} = 89$	$\frac{3.94}{4.42} = 89$	$\frac{1.11}{1.24} = 90$	$\frac{2.49}{2.74} = 91$	$\frac{1.38}{1.50} = 92$	$\frac{1.45}{1.68} = 86$
J.	"	168.2	59.6	1.68	$\frac{3.88}{3.36} = 115$	$\frac{5.21}{4.67} = 112$	$\frac{1.33}{1.31} = 102$	$\frac{3.41}{2.90} = 118$	$\frac{2.08}{1.59} = 131$	$\frac{1.80}{1.77} = 102$
H.	"	159.6	52.1	1.52	$\frac{3.17}{3.04} = 104$	$\frac{4.44}{4.22} = 105$	$\frac{1.27}{1.18} = 108$	$\frac{2.78}{2.62} = 106$	$\frac{1.51}{1.44} = 105$	$\frac{1.66}{1.60} = 104$
C.	"	165.0	53.0	1.58	$\frac{3.11}{3.16} = 98$	$\frac{4.86}{4.39} = 111$	$\frac{1.75}{1.23} = 142$	$\frac{3.23}{2.72} = 119$	$\frac{1.48}{1.49} = 99$	$\frac{1.63}{1.67} = 98$
Ho.	"	167.1	52.8	1.59	$\frac{3.83}{3.18} = 120$	$\frac{5.28}{4.41} = 120$	$\frac{1.45}{1.24} = 117$	$\frac{2.97}{2.74} = 108$	$\frac{1.52}{1.50} = 101$	$\frac{2.31}{1.68} = 137$
Mean value..........					104 ± 3	105 ± 2	106 ± 4	104 ± 3	102 ± 3	107 ± 4

TABLE VI.

Comparison of Observed to Calculated Lung Volumes in Twelve Patients with Heart Disease.

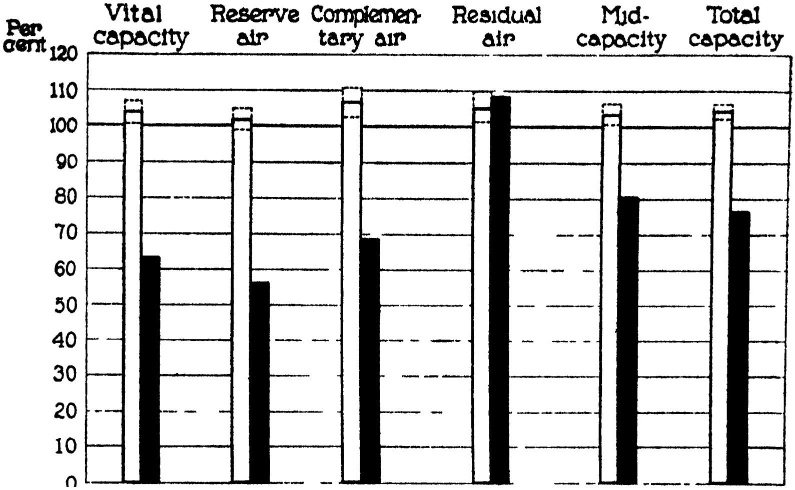
Date.	No.	Sex.	Height.	Weight.	Surface area.	Vital capacity $\left(\frac{\text{Observed}}{\text{Calculated}} = \text{per cent}\right)$.	Total capacity $\left(\frac{\text{Observed}}{\text{Calculated}} = \text{per cent}\right)$.	Residual air $\left(\frac{\text{Observed}}{\text{Calculated}} = \text{per cent}\right)$.	Middle capacity $\left(\frac{\text{Observed}}{\text{Calculated}} = \text{per cent}\right)$.	Reserve air $\left(\frac{\text{Observed}}{\text{Calculated}} = \text{per cent}\right)$.	Complementary air $\left(\frac{\text{Observed}}{\text{Calculated}} = \text{per cent}\right)$.	Diagnosis.
1922			*cm.*	*kg.*	*sq. m.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
Aug. 2	1	M.	167.7	57.6	1.66	$\frac{1.63}{4.15} = 39$	$\frac{3.44}{5.76} = 60$	$\frac{1.81}{1.61} = 112$	$\frac{2.51}{3.57} = 70$	$\frac{0.70}{1.96} = 36$	$\frac{0.93}{2.19} = 42$	Mitral stenosis and insufficiency; aortic insufficiency.
" 4						1.84 = 44	3.68 = 64	1.84 = 114	2.50 = 70	0.66 = 34	1.19 = 54	
Nov. 6						2.97 = 72	4.81 = 84	1.84 = 114	2.96 = 83	1.12 = 57	1.85 = 84	
Dec. 2						3.43 = 83	4.91 = 85	1.48 = 92	3.03 = 85	1.55 = 79	1.88 = 86	
June 26	2	"	170.8	57.1	1.67	$\frac{2.69}{4.18} = 64$	$\frac{5.12}{5.81} = 88$	$\frac{2.43}{1.64} = 148$	$\frac{3.33}{3.60} = 92$	$\frac{0.90}{1.97} = 46$	$\frac{1.79}{2.21} = 81$	Mitral stenosis and insufficiency; auricular fibrillation.
" 27						2.98 = 71	5.15 = 89	2.17 = 132	3.32 = 92	1.15 = 58	1.83 = 83	
Aug. 1						3.33 = 80	5.44 = 94	2.11 = 129	3.29 = 91	1.18 = 60	2.15 = 97	
Nov. 21	3	"	175.5	67.3	1.82	$\frac{2.06}{4.55} = 45$	$\frac{4.15}{6.32} = 66$	$\frac{2.09}{1.77} = 118$	$\frac{2.91}{3.92} = 74$	$\frac{0.82}{2.15} = 38$	$\frac{1.24}{2.40} = 52$	Mitral stenosis and insufficiency; aortic insuffi-

Dec. 16						$3.14 = 69$	$5.18 = 82$	$2.04 = 115$	$3.40 = 87$	$0.91 = 42$	$1.78 = 74$	ciency; auricular fibrillation
Nov. 6						$3.43 = 75$	$5.35 = 85$	$1.92 = 108$	$3.60 = 92$	$1.68 = 78$	$1.75 = 73$	
Aug. 7	4	M.	164	76.5	1.84	$\frac{3.01}{4.60} = 65$	$\frac{5.16}{6.39} = 81$	$\frac{2.15}{1.79} = 120$	$\frac{2.96}{3.96} = 75$	$\frac{0.81}{2.17} = 37$	$\frac{2.20}{2.43} = 91$	Mitral stenosis and insufficiency; aortic insufficiency; auricular fibrillation.
June 29						$3.18 = 69$	$5.06 = 79$	$1.88 = 105$	$2.86 = 72$	$0.98 = 45$	$2.20 = 91$	
	5	F.	150.6	30.1	1.18	$\frac{1.38}{2.36} = 59$	$\frac{2.27}{3.28} = 69$	$\frac{0.89}{0.92} = 97$	$\frac{1.62}{2.03} = 80$	$\frac{0.73}{1.11} = 66$	$\frac{0.65}{1.25} = 52$	Mitral insufficiency; auricular fibrillation.
	6	"	158.3	41.8	1.37	$\frac{1.77}{2.74} = 65$	$\frac{2.84}{3.80} = 75$	$\frac{1.07}{1.06} = 101$	$\frac{1.90}{2.36} = 81$	$\frac{0.83}{1.29} = 64$	$\frac{0.94}{1.45} = 65$	Mitral stenosis and insufficiency; auricular fibrillation.
	7	"	159.7	53.0	1.53	$\frac{2.58}{3.06} = 84$	$\frac{4.10}{4.25} = 97$	$\frac{1.52}{1.19} = 128$	$\frac{2.70}{2.64} = 102$	$\frac{1.18}{1.45} = 81$	$\frac{1.04}{1.62} = 86$	Mitral stenosis and insufficiency; aortic insufficiency.
	8	"	163.0	48.5	1.51	$\frac{1.75}{3.02} = 58$	$\frac{2.67}{4.19} = 64$	$\frac{0.92}{1.17} = 79$	$\frac{1.77}{2.60} = 68$	$\frac{0.85}{1.43} = 59$	$\frac{0.90}{1.59} = 57$	Mitral insufficiency; auricular fibrillation.
Oct. 26	9	"	150.5	44.0	1.37	$\frac{1.17}{2.74} = 43$	$\frac{2.34}{3.81} = 61$	$\frac{1.17}{1.07} = 109$	$\frac{1.63}{2.36} = 69$	$\frac{0.46}{1.29} = 36$	$\frac{0.71}{1.45} = 49$	Mitral stenosis and insufficiency; auricular fibrillation.

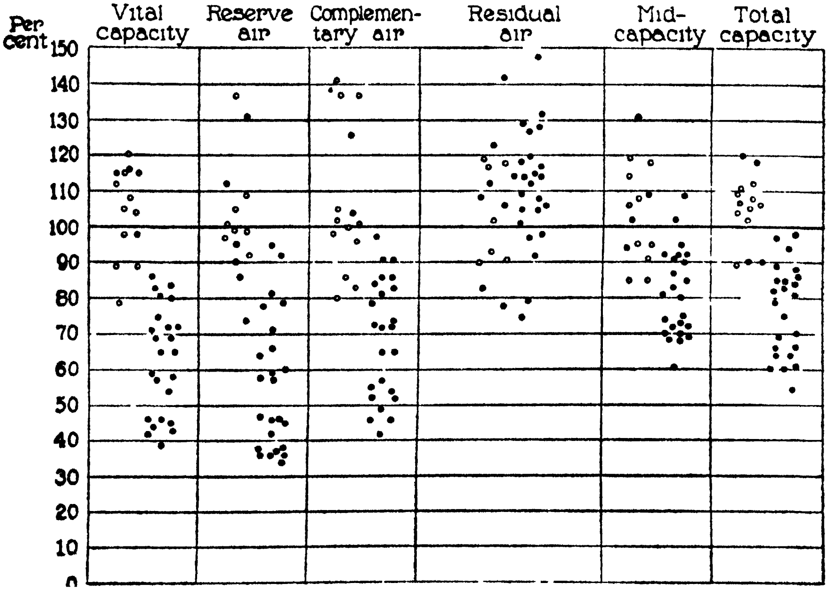
TABLE VI—*Concluded.*

Date.	No.	Sex.	Height	Weight.	Surface area.	Vital capacity. $\left(\frac{\text{Observed}}{\text{Calculated}} = \text{per cent}\right)$.	Total capacity $\left(\frac{\text{Observed}}{\text{Calculated}} = \text{per cent}\right)$.	Residual air $\left(\frac{\text{Observed}}{\text{Calculated}} = \text{per cent}\right)$.	Middle capacity $\left(\frac{\text{Observed}}{\text{Calculated}} = \text{per cent}\right)$.	Reserve air $\left(\frac{\text{Observed}}{\text{Calculated}} = \text{per cent}\right)$.	Complementary air $\left(\frac{\text{Observed}}{\text{Calculated}} = \text{per cent}\right)$.	Diagnosis.
1922			*cm.*	*kg.*	*sq. m.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
Oct. 28						1.26 = 46	2.51 = 66	1.25 = 117	1.71 = 72	0.46 = 36	0.80 = 55	
Nov. 2						1.56 = 57	2.68 = 70	1.12 = 105	1.73 = 73	0.61 = 47	0.95 = 65	
Nov. 1	10	F	162.0	57.6	1.62	$\frac{2.32}{3.24} = 72$	$\frac{3.75}{4.50} = 83$	$\frac{1.43}{1.26} = 114$	$\frac{2.51}{2.79} = 90$	$\frac{1.08}{1.53} = 71$	$\frac{1.24}{1.71} = 72$	Mitral stenosis and insufficiency; auricular fibrillation.
Dec. 2						2.64 = 81	3.88 = 86	1.24 = 98	2.65 = 95	1.41 = 92	1.23 = 72	
Aug. 24						2.80 = 86	4.40 = 98	1.60 = 127	3.05 = 109	1.45 = 95	1.35 = 79	
	11	"	158.5	45.6	1.39	$\frac{1.16}{2.78} = 42$	$\frac{2.30}{3.86} = 60$	$\frac{1.14}{1.08} = 106$	$\frac{1.63}{2.40} = 68$	$\frac{0.49}{1.31} = 37$	$\frac{0.67}{1.47} = 46$	Mitral stenosis and insuffiency; auricular fibrillation.
	12	"	147.3	53.5	1.45	$\frac{1.33}{2.90} = 46$	$\frac{2.20}{4.06} = 54$	$\frac{0.87}{1.16} = 75$	$\frac{1.50}{2.50} = 60$	$\frac{0.63}{1.37} = 46$	$\frac{0.70}{1.53} = 46$	Mitral stenosis and insufficiency; auricular fibrillation.
Mean value.						63	77	109	81	56	69	

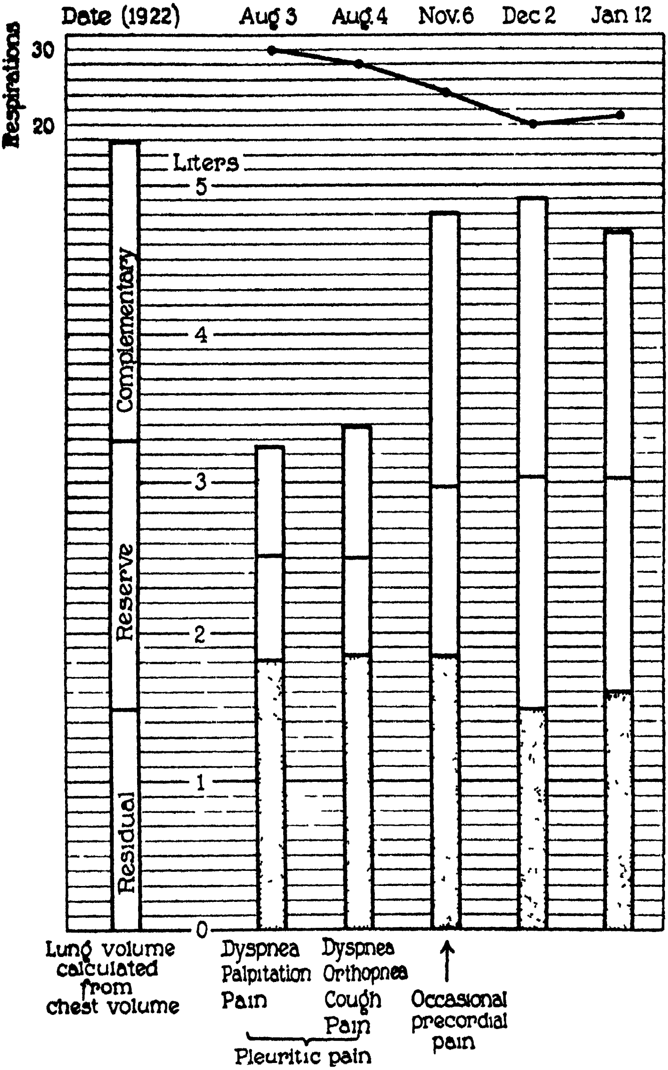
Where several determinations are given for the same patient they were made during progressive improvement from the decompensated toward the compensated state.



TEXT-FIG 5. Average percentage relation of observed to calculated lung volumes in fourteen normal subjects and twelve patients with heart disease. The black columns represent the percentage relation in the patients; the blank columns in the normals. The portion of the blank columns enclosed by the broken lines represents the average deviation for each lung volume.

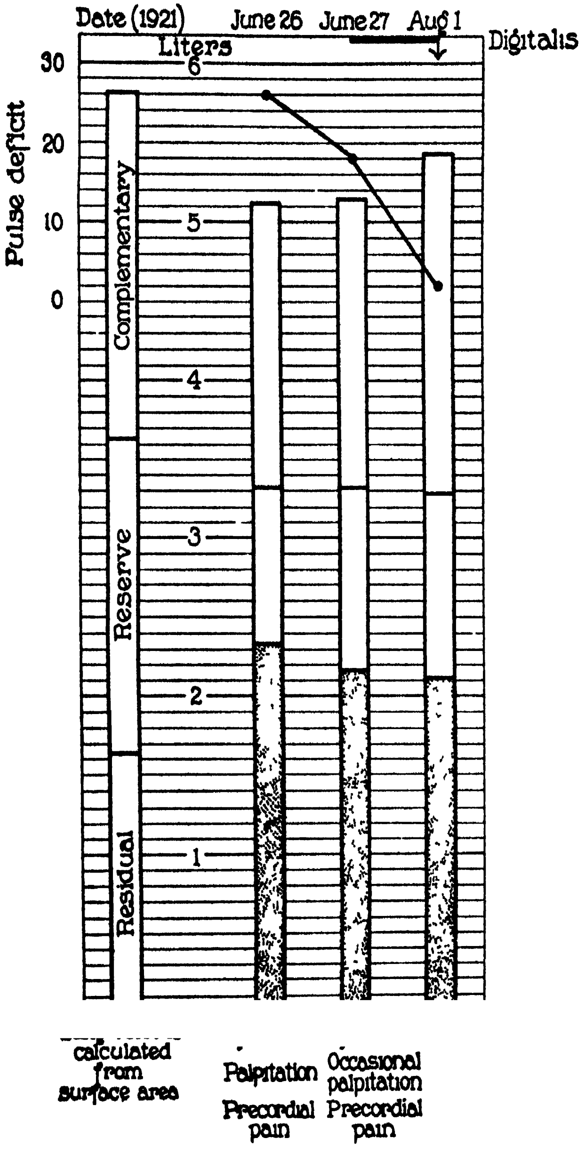


TEXT-FIG 6. Percentage relation of observed to calculated lung volumes in normal subjects and patients with heart disease. The black dots represent the percentage relation in the patients, the others the relation in normal individuals.

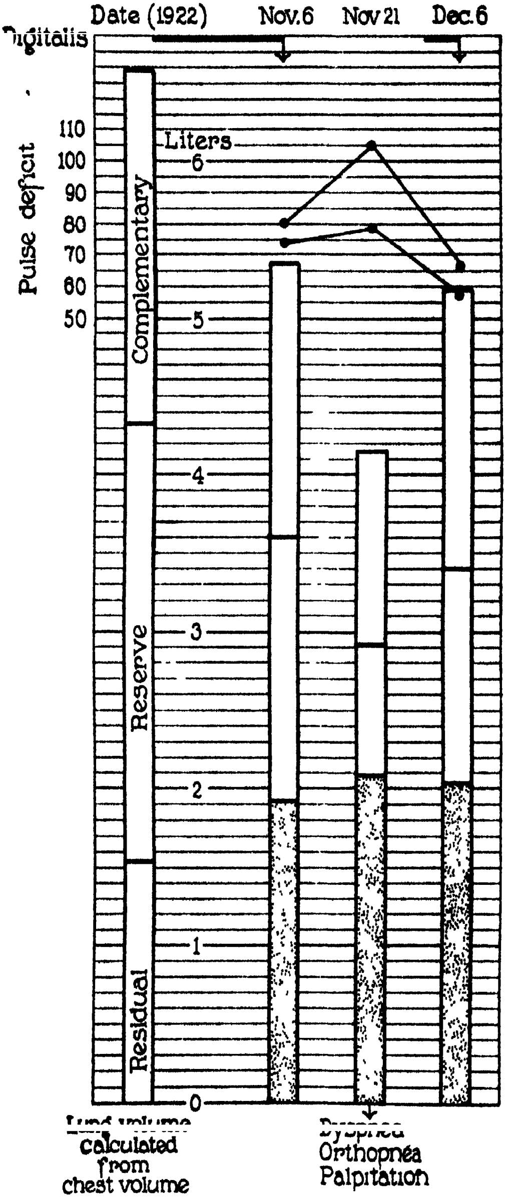


In Text-figs. 7 to 13 the first column represents the estimated lung volumes calculated from chest measurements after the method of Lundsgaard (11) or from the surface area.

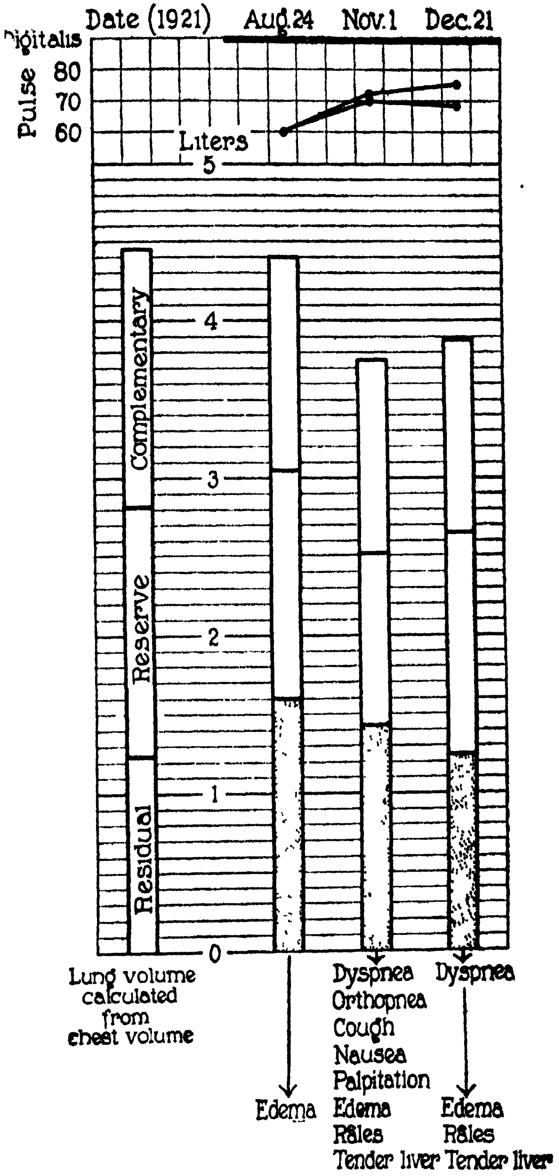
Text-Fig. 7. Case 1. Mitral stenosis and insufficiency; aortic insufficiency. Alterations in lung volume. The low total capacity found at the first two observations was associated with pleuritic pain which prevented the patient from taking a deep breath.



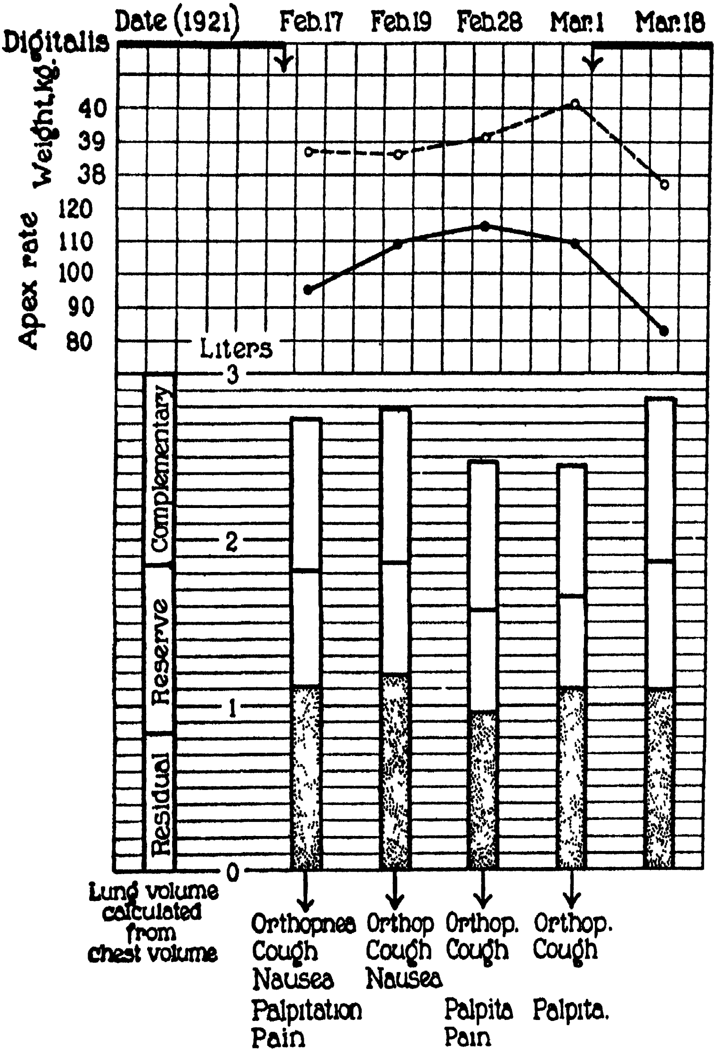
TEXT-FIG. 8. Case 2. Mitral stenosis and insufficiency; auricular fibrillation. Alterations in lung volume.



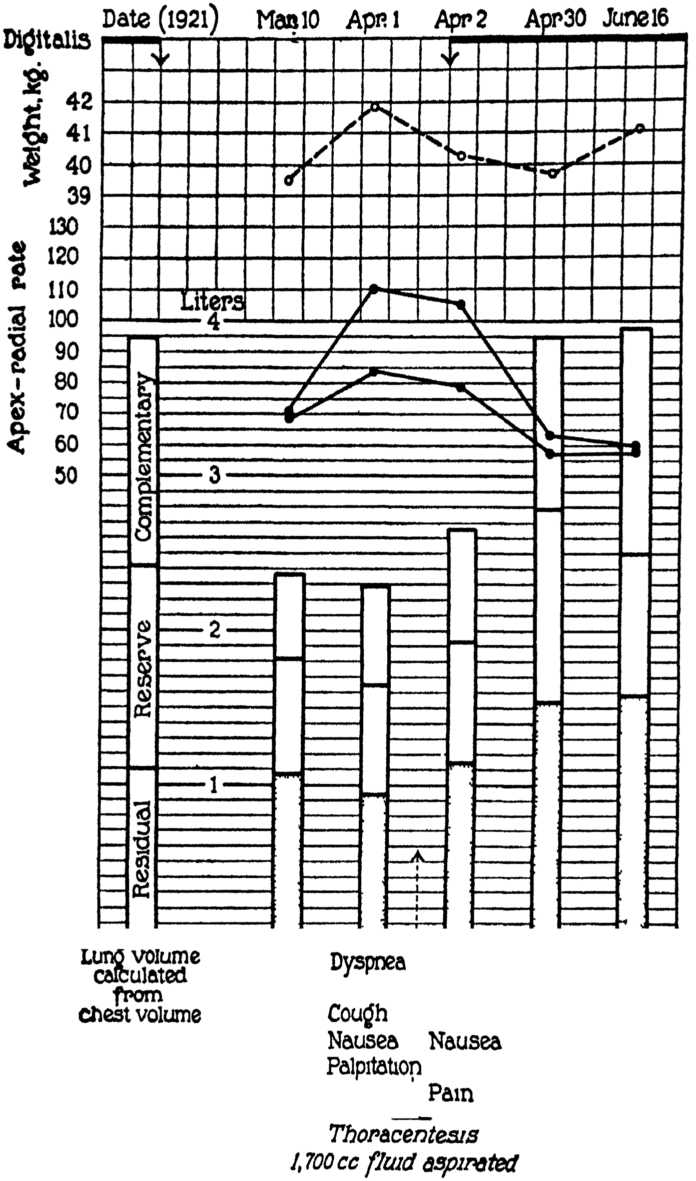
Text-Fig. 9. Case 3. Mitral stenosis and insufficiency; aortic insufficiency; auricular fibrillation. Alterations in lung volume.



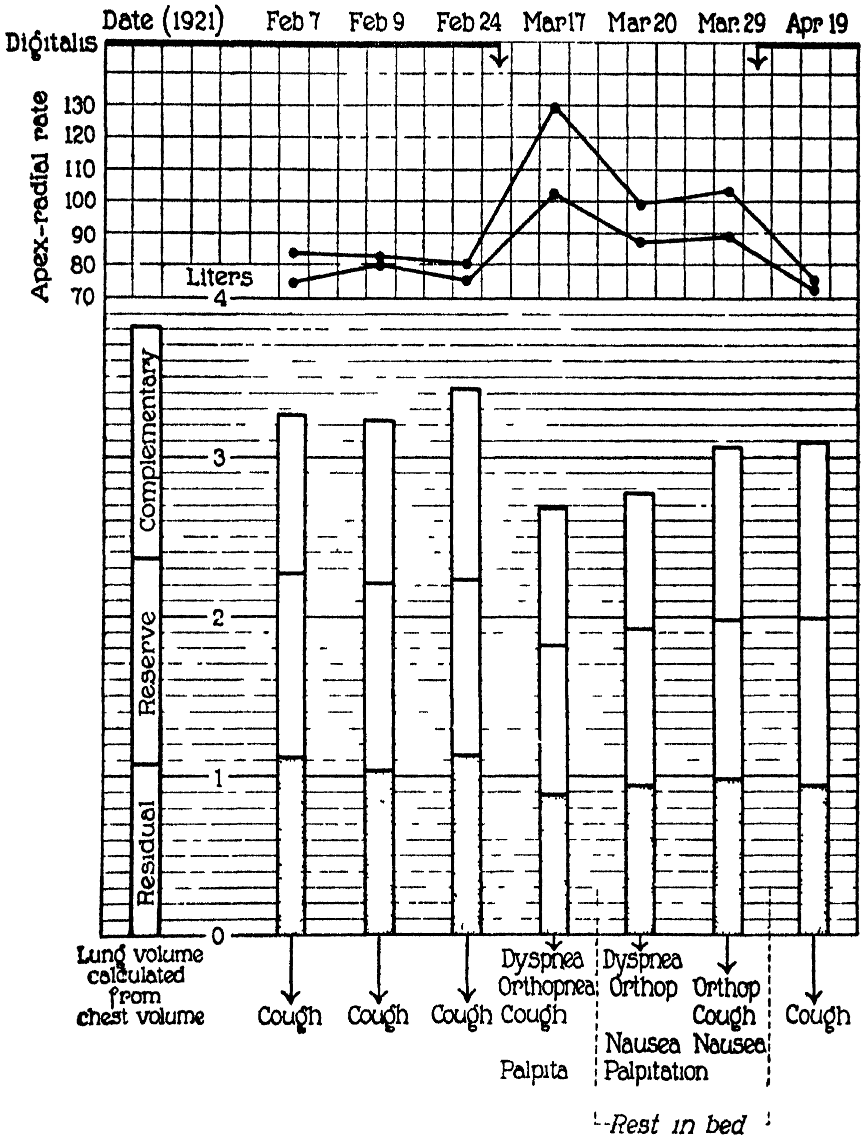
TEXT-FIG. 10. Case 10. Mitral stenosis and insufficiency; auricular fibrillation. Alterations in lung volume.



TEXT-FIG. 11. Case 6. Mitral stenosis and insufficiency; auricular fibrillation. Alterations in lung volume.



TEXT-FIG. 12. Case 13. Mitral stenosis and insufficiency; auricular fibrillation. Alterations in lung volume.



TEXT-FIG. 13. Case 14. Mitral stenosis and insufficiency; auricular fibrillation; aortic insufficiency. Alterations in lung volume.

or apex-radial rate, the curves employed in the individual cases being those which indicate changes in the patient's condition. Where no symptoms were recorded none were present. The symptoms particularly looked for were dyspnea, orthopnea, cough, palpitation, precordial or hepatic pain, nausea, and headache. Digitalis therapy is indicated by the broken horizontal line on the upper border of the charts. Dosage is not recorded, but in every instance sufficient quantities of the drug (digitan, Merck) were given to accomplish marked subjective improvement, decrease in apex rate, diminution of pulse deficit, increased fluid elimination, and loss of weight. A careful physical examination was made at the time the lung volume was determined. This included x-ray examination of heart and lungs.

Case 1.—L. C. (Text-fig. 7); male, age 24 years. Diagnosis: Chronic cardiac valvular disease; mitral insufficiency and stenosis; aortic regurgitation.

The patient had intermittent attacks of cardiac pain associated with elevation of temperature. Lung volume determinations were made during and after these attacks. During compensation the patient was able to fulfill the duties of a laboratory technician.

Case 2.—J. G. (Text-fig. 8); male, age 45 years. Diagnosis: Chronic cardiac valvular disease; mitral stenosis and regurgitation; cardiac arrhythmia; auricular fibrillation.

The first lung volume determination was made when the patient was suffering from signs and symptoms of heart failure; the second, a day later when there was subjective improvement; and the third, a month later when, after rest and digitalis, there was great improvement, the patient being able to mount one flight of stairs without discomfort.

Case 3.—T. S. (Text-fig. 9); male, age 24 years. Diagnosis: Chronic cardiac valvular disease; mitral stenosis and insufficiency; aortic insufficiency; cardiac arrhythmia; auricular fibrillation.

The first lung volume determination was made after a month's rest and digitalis therapy. The second shows the change concomitant with disappearance of the digitalis effect, and the third, the result of further digitalization. At this time the patient was free from symptoms and able to walk about the ward comfortably.

Case 10.—R. F. (Text-fig. 10); female, age 30 years. Diagnosis: Chronic cardiac valvular disease; mitral stenosis and regurgitation; cardiac arrhythmia; auricular fibrillation.

The first lung volume determination was made when the patient was relatively free from symptoms and able to walk about, though she was forced to live a much restricted life. 2 months later determinations were again made shortly after a break in compensation which required readmission to the hospital. Again 2 months later, after rest and digitalis therapy, a third determination was made. The patient's condition was improved, but compensation had not been restored. There was still dyspnea and râles were present at the bases.

Case 6.—F. S. (Text-fig. 11); female, age 16 years. Diagnosis: Chronic cardiac valvular disease; mitral stenosis and insufficiency; cardiac hypertrophy and dilatation; cardiac arrhythmia; auricular fibrillation.

Lung volume determinations were made at five different periods; the first four, while the effect of previous digitalis therapy was disappearing; the fifth, when the patient was again in a relatively compensated state as the result of further digitalization. At the time of the last observation the patient was sitting up in a wheel chair 2 hours daily. She was not fit for greater exertion.

Case 13.—L. C. (Text-fig. 12); female, age 42 years. Diagnosis: Chronic cardiac valvular disease; mitral stenosis and insufficiency; hydrothorax; cardiac arrhythmia; auricular fibrillation.

Lung volume studies were made at various intervals from March to June. Between the second and third observations, when the patient was not receiving digitalis, 1,700 cc. of fluid were aspirated from the pleural cavities. At this time there was evidence of adherent pleura which may account for the relatively slight increase in lung capacity after withdrawal of the fluid. The effect of rest and digitalis will be seen in the fourth and fifth lung volume determinations. There was gradual increase of vital capacity, total capacity, and residual air during convalescence.

Case 14.—R. S. (Text-fig. 13); female, age 51 years. Diagnosis: Chronic cardiac valvular disease; mitral stenosis and insufficiency; aortic insufficiency; cardiac arrhythmia; auricular fibrillation.

Lung volume determinations were made between February 7 and April 19, 1921. Determinations were made at seven different periods. During the first three the patient was under the influence of digitalis. The fourth, fifth, and sixth determinations were made while the patient was not receiving the drug. The increase in lung volume between the fifth and sixth determinations may be attributed to the effect of complete rest in bed even without digitalis. The seventh determination was made with the patient again digitalized, fairly free from symptoms, except for a cough, able to walk about on the level without dyspnea, though still weak, and at time suffering from palpitation. During the periods when no digitalis was given the patient had symptoms of cardiac insufficiency with râles at the bases, hepatic engorgement, and rapid heart rate with a wide pulse deficit. The changes occurring in this case were in the nature of a decrease in all lung volumes during the period of decompensation.

Simple inspection of these charts shows, as has been pointed out before, that in the individual case the absolute vital capacity, mid-capacity, and total capacity diminished during the progress of decompensation, and increased during restoration of the compensated state. The terms compensation and decompensation, it is realized, are rough when used in an absolute sense to define a patient's status; but when used in a relative sense to define a change in condition towards or away from heart failure, they become serviceable words. The charts also show that in most of the patients with heart disease, irrespective of the state of compensation, the values for these volumes are lower than those calculated for similar normal individuals.

No constant relation was determined between compensation and decompensation and the absolute values found for the residual air. But during the periods of decompensation these values for residual air became either larger or smaller. In the first three cases the residual air increased with onset of heart failure, in the last four it diminished. At the periods when compensation was best, that is when the total lung volume was largest, the values determined for the residual air were always larger than those calculated for similar normal individuals.

In other words, from these observations it appears that in a patient with heart disease the residual air volume is larger during compensation than is normal for a person of the same size. During decompensation the residual air volume may become still greater or it may diminish even below that for a normal individual.

These results, confusing as they may appear, are susceptible of what seems to be a rational interpretation. With the gradually increasing pressure in the pulmonary circuit resulting from a lesion of the mitral valve and the myocardial insufficiency, the vascular bed of the lungs becomes surcharged with blood. This gives to the lungs a stiffness (von Basch (17) (*Lungenstarre*)), analogous to that characteristic of erectile tissue. Forced expiration is therefore met with resistance, with the result that a given expiratory force does not produce the collapse which would occur were the lungs in their normal, spongy, elastic state. The result is an increase in the residual air. During this early stage of decompensation the total lung capacity may be normal, as in Subject 6 (Text-fig. 11), Subject 10 (Text-fig. 10), and Subject 13 (Text-fig. 12); and the reduction in vital capacity at that time may be associated with an increase in residual air alone. With the progress of heart failure and increasing pressure in the pulmonary veins the increase in residual air may become still greater, as in Subject 1 (Text-fig. 7), Subject 2 (Text-fig. 8), and Subject 3 (Text-fig. 9). At this time the same condition of rigidity which prevents forcible collapse of the lung may prevent forcible expansion, and reduction of the vital capacity may then depend upon reduction of the complementary as well as of the reserve air. With advancing heart failure and appearance of pulmonary edema and the structural changes found in chronic passive congestion, the air spaces of the lungs may be encroached upon. In this condition the residual air during decompensation may be diminished. Such a change is shown in Subject 6 (Text-fig. 11), Subject 10 (Text-fig. 10), Subject 13 (Text-fig. 12), and Subject 14 (Text-fig. 13). During this stage the enlarged heart and the engorged abdominal viscera may also operate to decrease the vital capacity and correspondingly affect the total capacity. This interpretation is for the most part in accord with that recently published by Lundsgaard (11). The fact that heart failure may result in either increase or decrease in residual air explains the confusion in which the subject has been shrouded, the confusion being aggravated by the fact that in some cases the residual air is normal in volume. This latter condition depends upon a neutralization of opposite effects; namely, those resulting from stiffness and those produced by edema.

It is obviously difficult to draw conclusions as to the functional significance of anatomical changes. This is especially true in the present instance since in individual cases in which changes in lung volume occur or in which abnormal lung volumes are found, the character of the anatomical alteration on which the volume changes depend can only be assumed by deduction from other facts and cannot be directly demonstrated.

Two distinct and important processes are concerned in the phenomenon of external respiration: first, the mechanical bellows movements of the chest by which the diffusion of O_2 into the alveolar air and CO_2 outward is facilitated; and second, the passage of O_2 into the capillary blood and CO_2 from the blood. The latter depends upon (1) the composition of the alveolar air, (2) the state of the blood, and (3) the anatomical character of the alveolar walls. Changes in the volume of air in the lung in the different phases of respiration may directly or indirectly affect all these factors.

The significance of changes in vital capacity has been well and fully discussed by Peabody and his associates. In general it may be said that a reduced vital capacity indicates a reduction in pulmonary reserve in the sense that the vital capacity sets the limit to which the minute volume of pulmonary ventilation can be raised in response to metabolic demands.

The significance of changes in total lung volume and in mid-capacity is not so clear. Changes in total lung volume in the individual case may depend upon one or more of a number of factors. Encroachment upon the chest space by other organs or tissue may give rise to a decrease in total lung volume. Decreased elasticity or stretching of the lung may give rise to increase in total volume as in emphysema. On the other hand, decreased elasticity associated with stiffness or thickening of the parenchyma may be associated with a decrease in lung volume.

In addition to these purely anatomical explanations of changes in total lung volume, however, there are undoubtedly numerous factors which regulate not only the rate and depth of the respiratory movements of the chest, but also determine the character of the movements and the degree of distention of the lungs during the various respiratory stages. The degree of distention is best characterized by the mid-position, and numerically established by determining the mid-capacity. In patients with heart disease, both during the state of compensation and during the state of decompensation, we have found the mid-capacity as well as total capacity to be usually low, as compared with values for similar normal individuals, the decrease progressing with decompensation.

Bohr (2) found that in normal individuals increased metabolic demands, as in exercise, were followed by increase in mid-capacity. He, therefore, concluded that in normal individuals a small mid-capacity indicated functional fitness (*Functionsfähigheit*). He inferred that increase in mid-capacity represented a favorable response to increased strain and explained why such a response is of advantage by assuming that with increased distention of the lung parenchyma the resistance to blood flow through the capillaries became less. For this assumption there is indeed considerable experimental evidence such as that of Schafer (18).

Rubow (5) found in patients with heart disease that the mid-capacity, considered as a percentage of the total capacity, was increased. In view of Bohr's hypothesis he concluded that this relative increase in mid-capacity in these patients represented a favorable response to increased demands.

The present studies, however, indicate that in patients with heart disease, although the mid-capacity in relation to total capacity is usually increased, the absolute mid-capacity as determined by comparison with a normal individual or as determined in the same individual in various stages of decompensation is decreased.

In view of the findings it is difficult, therefore, to look upon the alteration in mid-capacity in patients with heart disease as a favorable response. It seems more likely that this decrease in mid-capacity, which has been found, is an indication of intrapulmonary vascular distention and therefore associated with increased pressure and decreased rate of flow in the pulmonary circulation. Instead, therefore, of an increased mid-capacity and consequent favorable physiological state in the lung as Rubow thought, there is actually a lowered mid-capacity which is probably unfavorable.

SUMMARY AND CONCLUSIONS.

The lung volumes in a group of individuals suffering from chronic cardiac disease have been studied by a method which is applicable to patients suffering from dyspnea. In a number of instances the same patients were investigated during various stages of decompensation and compensation. The values found have been compared with those determined in a group of normal subjects.

Lung volumes have been considered from three points of view: (1) relative lung volumes or subdivisions of total lung volume expressed as percentage of total lung volume; (2) the absolute lung volumes of patients with heart disease have been compared with lung volumes calculated for normal individuals having similar surface areas or chest measurements; and (3) in individual cases absolute lung volumes have been measured in various stages of compensation and decompensation.

(1) In patients with heart disease it has been observed that the vital capacity forms a portion of the total lung volume relatively

smaller than in normal individuals, and that the mid-capacity and residual air form relatively larger portions. When the patient progresses from the compensated to the decompensated state these changes become more pronounced.

(2) When the absolute lung volumes determined for patients are compared with volumes of the same sort, as calculated for normal individuals of the same surface areas and chest measurements, the following differences are found. The vital capacities are always smaller in the patients and the volumes of residual air are always larger. There is a tendency for middle capacity and total capacity to be smaller, though, when the patients are in a compensated state, these volumes may approximate normal.

(3) When decompensation occurs the absolute lung volumes undergo changes as follows: (*a*) vital capacity, mid-capacity, and total capacity decrease in volume; and (*b*) the residual air may either increase or decrease according to the severity of the state of decompensation.

The significance of these changes has been discussed and an explanation offered for the occurrence of a residual air of normal volume in patients with heart disease. It results from a combination of two tendencies working in opposite directions: one to increase the residual air—stiffness of the lungs (*Lungenstarre*); the other to decrease it—distended capillaries (*Lungenschwellung*), edema, round cell infiltration.

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EXPERIMENTAL STUDIES IN DIABETES.

SERIES II. THE INTERNAL PANCREATIC FUNCTION IN RELATION TO BODY MASS AND METABOLISM.

11. THE RELATION OF THE ADRENALS TO DIABETES.

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In continuance of an earlier investigation,[1] the writer performed experiments in the years 1917 and 1918 concerning possible relationships between the pancreas and adrenals in regard to the etiology or symptoms of diabetes. The three lines of experiment consisted in partial epinephrectomy, fat feeding in connection with epinephrin injections, and epinephrin injections in partially depancreatized dogs.

1. Partial Epinephrectomy.

The low blood sugar of patients with Addison's disease and the still more marked hypoglycemia of totally epinephrectomized animals were observed by authors previously quoted[1,2,3] and confirmed by practically all later workers.[4,5,6,7] It was also demonstrated that the hyperglycemia and glycosuria ordinarily following pancreatectomy are completely prevented when both adrenals are removed at or near the same time[8,9,10] (but not when they are removed at the height of the diabetes[11,12]). Such observations furnished the chief support of the pluriglandular doctrine of diabetes. Subsequent work, however, has proved (a) that the conditions in adrenal deficiency in many respects resemble any other state of collapse;[13,14,15] (b) that the early death following bilateral epinephrectomy, as also the hypoglycemia and other symptoms, are due to loss of the cortical and not the medullary portion of the adrenals, because animals survive if they possess sufficient accessory cortical substance or if a sufficient portion of the cortex be spared;[1,16,17,18] (c) that carbohydrate metabolism is unaffected and diabetic and other forms of glycosuria occur as usual after operations which thus leave sufficient cortical tissue while removing the entire chromaffin tissue of the adrenals.[16,19,20 21] This evidence was less complete at the time when the experiments here to be presented were performed, but as the method was different from that used by others, the results may still be worth recording.

A series of experiments were performed by the removal of as much adrenal tissue as seemed compatible with life, and testing the possible influence upon carbohydrate metabolism. Intravenous glucose injections were chiefly employed, by the discontinuous method previously described,[22] to guard against any irregularities of absorption under these conditions. The other methods were the same as in former papers.

Dogs F6-83, F6-84 and G7-39 were normal animals which underwent removal of the greater part of the left adrenal and subsequently of the entire right adrenal, leaving only about one-sixth of the total adrenal tissue, and which received intravenous glucose tolerance tests a few days before the first operation and 2 or 3 weeks after the second operation. The results of these tests, shown in Table 1, indicate certainly no elevation of tolerance by the reduction of adrenal tissue. A lowering of assimilation might be suspected especially in dog F6-84, but all the differences are probably within accidental variations.

DOG E5-92.

Partial Epinephrectomy in Diabetes.

Female, mongrel, age 3 or 4 years, good condition; weight 16.75 kg. August 31, 1917, removal of pancreatic tissue weighing 29.6 gm. Remnant about main duct estimated at 3.4 gm. (1/9–1/10). High carbohydrate diets at first were necessary for glycosuria, but the diabetes was allowed to progress to a severe stage, so that by repeated tests the tolerance was found to be below 400 gm. of beef lung. Thus, on November 28, the weight was 12.5 kg.; the plasma sugar was 0.167 per cent. before feeding; 6 hours after feeding 400 gm. lung and 100 gm. suet it was 0.269 per cent., and the urine was 395 cc. with 2.4 per cent. sugar. After a fast-day to stop glycosuria, ⅔ to ¾ of the left adrenal was removed on November 30. The diet on the next day was 100 gm. lung and 100 gm. suet, and was increased gradually to 400 gm. lung and 100 gm. suet on December 4, when glycosuria returned. It ceased when the lung was reduced to 100 gm., returned with an increase to 300 gm. on December 8, and was continuous on 300 gm. lung and 100 gm. suet to December 12, with plasma sugars as high as 0.4 per cent.

December 12, at a weight of 11.7 kg., the entire right adrenal was removed by an operation which was very easy in the emaciated dog. Glycosuria was stopped by the one fast-day, and failed to return on the diet of 300 gm. lung and 100 gm. suet.

A sufficient reason was present in the cachexia and diarrhea of poorly digested food. On December 27 the weight was 11 kg., the plasma sugar 0.147 per cent. before feeding and 0.185 per cent. six hours after feeding. The weight and strength continued to fail, until on January 18, 1918, the dog weighed 9.5 kg. and was too feeble to stand. The plasma sugar was 0.042 per cent., the CO_2 capacity 34.7 vol. per cent.; acetone negative. At autopsy the pancreas remnant weighed 3.9 gm., and the results of the adrenal operations were confirmed. Microscopically, the pancreatic acini were empty but not involuted. Islands were present in fair size and number, but with vacuolation in a few cells. This, and some Armanni changes in the kidneys, indicated that hyperglycemia probably continued up to the terminal collapse. The viscera otherwise were negative.

TABLE 1.

Dogs receiving intravenous tolerance tests with 1.5 gm. Merck glucose per kg. per hour in 10% solution, 3 injections per hour for 3 hours, before and after partial epinephrectomy.

	Dog F6-83 Weight 9 Kg.			Dog F6-84 Weight 9 Kg.			Dog G7-39 Weight 11 Kg.		
	URINE		Plasma Sugar %	URINE		Plasma Sugar %	URINE		Plasma Sugar %
	Vol. cc.	Glucose %		Vol. cc	Glucose %		Vol. cc.	Glucose %	
Before Operation									
Before injection	25	0	0 109	18	0	0 086	15	0	0.118
End of 1st hour	52	3 70	0 159	50	2 63	0 175	10	2.00	0.167
“ “ 2nd “	58	2.86	0 152	26	2.80	0 179	5	2 80	0.137
“ “ 3rd “	124	2.83	0 218	90	1.50	0 104	148	0.26	0.232
1 hour after ending injections	38	Faint	0 102	63	Faint	0 075	44	Faint	0.085
2 hours after ending injections	12	“	0 114	25	“	0 089	66	0	0.131
After Operation									
Before injection	38	0	0 099	10	0	0 094	56	0	0.088
End of 1st hour	41	4 08	0.263	26	8 70	0 303	30	0 87	0.219
“ “ 2nd “	40	1 77	0 256	23	4 08	0 244	32	0.88	0.145
“ “ 3rd “	71	1.24	0.145	138	3 23	0 179	60	0 41	0.156
1 hour after ending injections	53	0 28	0.089	25	0.26	0 077	86	Faint	0.084
2 hours after ending injections	19	Faint	0 093	20	Faint	0.095	34	0	0 088

The cachexia and asthenia of this animal were suggestively like those of Addison's disease. Also as noticed in connection with the thyroid,[23] operations on different organs which separately are well borne sometimes produce fatal cachexia when combined. The suppression of glycosuria and partial reduction of hyperglycemia here are not appreciably different from the rule in any cachexia.[24] No benefit of the adrenal operations is perceptible, for the resulting condition was fatal, while many experiments in this and the preceding series have shown that with simple undernutrition the diabetes in such a case can be controlled and the animal kept alive apparently indefinitely.

2. *Fat Feeding in Relation to Epinephrin Glycosuria.*

It was asserted by Blum[25] and confirmed by Roubitschek[26] that when animals have fasted to such a point that a certain dose of epinephrin no longer causes glycosuria, the feeding of pure fat for several days causes them to be subject to glycosuria from the same dose. These writers interpreted their results as proof of the formation of sugar from fat. Eppinger, Falta and Rudinger[27] reported a similar finding, but were inclined to believe that the fat merely spared glycogen. Though these reports are inherently improbable, the experiments seemed worth repeating for two reasons: (a) It is generally accepted that fat tends to drive out glycogen from the liver, and it is thus conceivable that fat feeding might augment the effect of epinephrin; (b) Fat exerts a powerful influence upon diabetic glycosuria,[28,86] and a similar observation in connection with any other form of glycosuria would be of interest.

Numerous trials were made which need not be reported in detail, as they merely confirm Underhill's[29,30] conclusion that this form of experiment is unreliable; no uniformity of either hyperglycemia or glycosuria can be anticipated in the same animal or in different animals from the same dose of epinephrin. The influence of single or repeated fat feedings upon the sugar of blood or urine was negative throughout. This statement holds not only for normal dogs but also for those depancreatized so as to lower the tolerance nearly to the point of diabetes. A single experiment of this type will be given as an illustration.

Dog B2-00, normally weighting 14 kg., had been subjected to partial pancreatectomy to such an extent that the removal of approximately 2.5 gm. of tissue was subsequently found necessary to produce diabetes. Fasting was begun May 8, 1916, at a weight of 14.3 kg. May 15, at a weight of 12.9 kg., a subcutaneous injection of 4 cc. of Parke-Davis adrenalin solution (1/1000) was given in the fasting condition, with the result of hyperglycemia without glycosuria, as shown in Table 2.

May 17, the feeding of 100 gm. lard daily was begun. May 22, at a weight of 11.9 kg., 200 gm. lard was fed at 9 A. M., and at 2:45 P. M. an epinephrin injection given as before. There was still no glycosuria, and the difference in hyperglycemia was within the limits of accidental variation.

Plain fasting was then resumed. May 26, 300 gm. white clay was fed at 9 A. M., by mixing with water and molding in convenient lumps, each bolus being swallowed readily when placed in the back of the mouth. The purpose was to test any possible influence of mere fullness of the stomach. There was still no glycosuria from the adrenalin injected in the afternoon, and no important difference in the hyperglycemia.

With continuance of fasting to May 29, the weight was down to 10.9 kg., and the animal was distinctly weak. After the feeding of 500 gm. of beef lung, the same dose of adrenalin produced the highest hyperglycemia of the series, together with slight glycosuria. Though there were fewer calories and fewer grams of protein on this day than of fat on May 22, and though it was at a later stage of fasting, the sugar production from protein was nevertheless positive, in contrast to the negative results of fat.

TABLE 2.

Dog B2-00.

Weight 14 kg. Partially depancreatized, not quite diabetic.

Injection of 4 cc. adrenalin solution (1/1000) subcutaneously at 2:45 P. M. on 4 different days.

Time	PLASMA SUGAR %				Remarks
	May 15	May 22	May 26	May 29	
2:40 P. M.	0.123	0 095	0.105	0.092	May 15, fasting.
3:45 P. M.	0.126	0 110	0 104	. .	“ 22, fed 200 gm. lard.
4:45 P. M.	0.175	0 095	0 137	0 154	“ 26, “ 300 gm. white clay.
6:00 P. M	0 196	0 161	0.162	0.228	“ 29, “ 500 gm. lung.
7:30 P. M.	0 200	0 186	0 250	0.250	
10:30 P. M.	0 170	0 230	0 244	0 323	
Total glucose excreted, gm.	0	0	0	0 12	

3. *Epinephrin Injections in Relation to Diabetes.*

The experiments under this heading were planned from three points of view.

First, diabetic dogs are subject to hydropic degeneration of the islands of Langerhans from the functional overstrain of excessive diets.[31] If epinephrin is specifically antagonistic to the island function, it may perhaps produce morphologic changes in animals predisposed by suitable partial pancreatectomy.

Second, if, as alleged in pluriglandular speculations, the hormone of the islands normally acts as a "brake" upon sugar formation in the liver, and diabetes consists in excessive glycogenolysis by epinephrin due to the removal of this "brake," exaggerated effects should be produced by epinephrin injections in animals made diabetic by partial pancreatectomy.

Third, diabetes is characterized by excessive and persistent formation of sugar not merely from glycogen but also from protein. Several authors[32] have described increased excretion of nitrogen in fasting animals as a result of epinephrin injections. Differences of dosage and methods may explain various discrepancies. The most reasonable explanation is that after depletion of its glycogen by epinephrin the fasting organism undertakes to restore its glycogen reserve, and does so by using protein for the purpose. In this way the increased nitrogen excretion is merely a secondary phenomenon, and the general experience is that epinephrin under ordinary conditions of nutrition does not increase nitrogen loss. Observations on this subject in partially depancreatized dogs seemed desirable for two reasons: (a) to determine whether under these circumstances epinephrin will reproduce this important feature of diabetes, namely, glycosuria at the expense of increased protein destruction; (b) in addition for possible light upon a new question, namely, whether diabetic tissues are more easily subject to breakdown from various causes than the normal.

For this purpose, a series of diabetic dogs and normal controls were subjected to experiments as follows:

DOGS D4-77 AND D4-89.

Dog D4-77 was partially depancreatized January 19, 1917, leaving a remnant estimated at about one-ninth about the main duct. The mild diabetes which resulted was made severe by carbohydrate overfeeding, and the condition was then kept under control by protein-fat diet and undernutrition. Dog D4-89 was chosen as a normal control and subjected to the same conditions, except operation. The two dogs were then used for experiments with thyroid feeding, as previously described.[23] Following the thyroid period, injections of adrenalin were given as shown in the tables, first on the diet mentioned and later on fasting.

TABLE 3.

Dog D4-77.

Date 1917	Time	BLOOD		URINE				Body Weight kg.	Remarks
		Plasma Sugar mg per 100 cc	CO_2 vol. %	Vol. cc.	Sugar gm.	Acetone qual.	Total-N gm.		
May 11	12:30 P.M.	72	50.4	395	Neg.	Neg.	3 20	8 5	3:30 P.M. injected intravenously 10 cc. adrenalin 1/100,000.
									4:00 P. M. injected 20 cc. adrenalin 1/100,000.
									5:00 P. M. " " " "
	6:00 P. M.	130	....	...	....	.....	.	. .	5:30 P. M. fed 250 gm. lung, 100 gm. suet.
" 12		...	....	708	"	"	3 28	....	12:00 M, injected 1 cc. adrenalin intraperitoneally 1/1000.
									6:00 P. M. fed the same diet.
" 13		...	....	570	"	"	3 24	....	11:00 A.M. injected 2 cc. adrenalin 1/1000.
									5:30 P. M. fed same diet forcibly, all vomited.
" 14	9:55 A.M.	119	....	378	"	"	2 96	....	Injected 3 cc. adrenalin (1/1000) not fed.
" 15	11:15 A.M.	119	53 8	662	"	Faint	3 60	....	" " " " " "
" 16	10:00 A.M.	109	55.7	520	"	"	3 72	7.5	" 5 cc. " " " "
	1:45 P.M.	278	56.0	...	....	.....	....	....	....
	6:30 P.M.	167	....	...	....	.....	....	....	....
" 17		...	....	153	"	Neg.	2 02	....	No adrenalin today. Not fed.
" 18		...	....	316	"	Faint	3 20	7.35	" " " " "
" 19	10:00 A.M.	102	58.9	460	"	Slight	2.75	7.5	" " " " "

Total N output for 9 days	27.97
Average N output per day	3.11
N output per kg. per day	0.36

TABLE 4.

Dog D4-89.

Date 1917	Time	BLOOD		URINE				Body Weight kg.	Remarks
		Plasma Sugar mg. per 100 cc.	CO₂ vol. %	Vol. cc.	Sugar gm.	Acetone qual.	Total-N gm.		
May 11	12:30 P.M.	80	59 5	206	Neg.	Neg.	2 30	9 8	3:30 P. M. injected intravenously 10 cc. adrenalin 1/100,000. 4:00 P M. injected 20 cc. adrenalin 1/100,000. 5:00 P. M. " " " "
	6:00 P. M.	105	61 4	...	.	.		. . .	5:30 P. M. fed 250 gm. lung, 100 gm. suet.
" 12		...	....	310	"	"	5 12	. . .	12:00 M. injected 1 cc. adrenalin intraperitoneally 1/1000. 6:00 P. M. fed same diet.
" 13		...	. .	330	"	"	4 24	....	11:00 A. M. injected 2 cc. adrenalin 1/1000. 5:30 P. M. fed same diet.
" 14	9:50 A. M.	79	.	386	"	"	7.12	....	Injected 3 cc. adrenalin (1/1000) not fed.
" 15	11:55 A M.	55	57 6	190	"	"	2 66	....	" " " " " "
" 16	10:00 A. M.	74	58 6	200	"	"	3 92	8.75	" 5 cc. " " " "
	1:45 P. M.	154	60 5	...	....	. . .	....	....	.. ..
	6:30 P. M.	80	....	...	....	.. ..	....	....	... .
" 17		...	.. .	154	"	"	3 20	....	No adrenalin today. Not fed.
" 18		..		230	"	"	1 48	8.3	" " " " "
" 19	10:00 A. M.	74	61 7	255	"	"	4 26	8.25	. . ..

Total N output for 9 day	34 30
Average N output per day	3 81
N output per kg. per day	0 39

Though dog D4-77 actually had diabetes of considerable severity, and was only kept sugar-free by close dietary restriction, no marked exaggeration of the effects of either intravenous or subcutaneous adrenalin doses was demonstrable. There was no glycosuria in either animal, and the differences in regard to hyperglycemia were not extreme. The nitrogen loss of the two dogs was also practically identical.

DOGS E5-00 AND E5-46.

Dog E5-00 was made severely diabetic by successive pancreatic operations on March 8 and April 3, 1917, the remnant about the main duct being estimated at between one-ninth and one-tenth of the gland. After the glycosuria had become well established, it was checked by fasting and low protein-fat diet. Dog E5-46 was kept on the same diet, as a normal control.

The severity of the diabetes in dog E5-00 is indicated by the glycosuria which resulted from the feeding of only 200 gm. beef lung and 100 gm. suet on May 29. A fast-day, followed by a day of suet only, raised the tolerance so that this quantity of protein was tolerated following June 1. The subsequent intraperitoneal adrenalin injections produced no glycosuria in the fasting normal dog and only trivial sugar excretion in the severely diabetic dog. The nitrogen excretion per kilogram happened to be identical in the two animals.

DOGS F6-07 AND F6-08.

These two animals were chosen as nearly equal in weight, and were partially depancreatized on December 6, 1917. The remnant communicating with the main duct was estimated in dog F6-07 as one-eleventh, and in dog F6-08 as one-twenty-first of the gland; i.e., under ordinary circumstances the diabetes would be more severe in the latter dog. By reason of fasting one day before operation and continuously afterwards, dog F6-08 remained free from glycosuria. There was rapid enlargement of the pancreas remnant, the weight of which was estimated at 1.4 gm. on December 6. On December 14, 0.5 gm. additional was removed, and on December 19 an additional 1.25 gm., and when the dog was chloroformed for autopsy on December 31 the remnant still weighed 1 9 gm. The pancreas specimens from all the operations and the autopsy were microscopically normal, except for doubtful thinning of cytoplasm in the island cells at the close, as though a slight tendency to exhaustion might be present.

The pancreas remnant in dog F6-07 was estimated at 2.5 gm., and at autopsy on December 16 was found to weigh 2.6 gm. The difference in hypertrophy as compared with dog F6-08 need not be attributed to adrenalin, as different dogs vary widely in this respect for unknown causes.[31] The pancreatic tissue at autopsy was microscopically normal except for slight but distinct vacuolation in a number of cells of all the islands.

Slight glycosuria resulted from the intraperitoneal adrenalin injections in dog F6-07. This effect was less than might have been anticipated from the severity of the potential diabetes, and the progressiveness characteristic of diabetes seemed to be absent. Excessive protein catabolism was also absent.

TABLE 5.

Dog E5-00.

Date 1917	Time	BLOOD		URINE				Body Weight kg.	Remarks
		Plasma Sugar mg. per 100 cc.	CO_2 vol. %	Vol. cc.	Sugar gm.	Acetone qual.	Total-N gm.		
May 29		...	....	463	4.00	Neg.	5.65	14.5	Fed 300 gm. lung, 100 gm. suet.
" 30		...	....	390	Faint	"	4.34	....	Not fed.
" 31		...	....	368	Neg.	"	7 56	....	Fed 200 gm. suet.
June 1		...	....	328	"	"	3 44	....	" 300 gm. lung, 100 gm. suet.
" 2		...	....	456	"	"	5 70	....	" " " 200 gm. "
" 3		...	....	432	"	"	5 75	....	" " " " "
" 4		...	....	810	"	"	8 04	....	Not fed.
" 5		...	....	512	"	"	3 24	....	Fed 300 gm. lung, 200 gm. suet.
" 6		...	....	470	"	"	5.85	....	" " " " "
" 7	6:00 P. M.	101	....	536	"	"	4.94	....	Not fed. Injected 2 cc. adrenalin intraperitoneally.
	9:00 P. M.	196	....	...	....	.....	....	....	....
" 8	10:30 A. M.	147	....	445	Faint	"	4 14	....	Not fed. Injected 4 cc. adrenalin intraperitoneally at 12:30 P. M.
	3:00 P. M.	346	....	...	....	.....	....	....	
	10:00 P. M.	159	....	...	....	.....	....	....	....
" 9	9:15 A. M.	161	....	208	2.20	"	3 30	13.3	Not fed. Injected 6 cc. adrenalin.
	12:00 M.	344	....	...	....	.....	....	....	....
" 10		...	....	348	Neg.	"	4 16	....	No adrenalin today.
" 11	1:30 P. M.	90	50.4	200	Faint	"	2.34	....	Not fed. Injected 8 cc. adrenalin intraperitoneally.
	6:00 P. M.	263	52.2	...	....	.....	....	....	....
" 12	9:30 A. M.	112	57.9	275	Neg.	"	2.41	....	No adrenalin today.
" 13		...	....	270	"	"	2.21	....	....
" 14	11:00 A. M.	90	....	432	"	"	4.08	...	... .
	8:00 P. M.	222	....	...	....	.....	....	....	....
Total N output for 17 days							77.15		
Average N output per day							4.54		
N output per kg. per day							0.31		

TABLE 6.

Dog E5-46.

Date 1917	Time	BLOOD		URINE				Body Weight kg.	Remarks
		Plasma Sugar mg. per 100 cc.	CO₂ vol. %	Vol. cc.	Sugar gm.	Acetone qual.	Total-N gm.		
May 29		...	....	199	Neg.	Neg.	6 68	15.5	Diet 300 gm. lung, 100 gm. suet.
" 30		...	....	210	"	"	7.90	.. .	Not fed.
" 31		...	....	128	"	"	....	15.35	Diet 200 gm. suet.
June 1		...	....	118	"	"	3 68	15.	Fed 300 gm. lung, 100 gm. suet.
" 2		...	....	145	"	"	4.72	15.25	" " " 200 gm. "
" 3		...	....	No Urine		.....	....	....	....
" 4		...	....	480	Neg.	Neg.	18.30	15.6	Not fed.
" 5		...	....	108	"	"	3.16	15 25	Fed 300 gm. lung, 200 gm. suet.
" 6		...	....	160	"	"	3 14	15.35	" " " " "
" 7	6:00 P. M.	65	....	342	"	"	7.44	....	Not fed. Injected 6 cc. adrenalin in peritoneum.
	9:00 P. M.	164	....	...	....	.....	....	15 55	....
" 8	10:30 A. M.	103	....	162	"	"	4.78	....	Not fed. Injected 4 cc. adrenalin in peritoneum.
	3:00 P. M.	151	....	...	....	.....	....	....	....
	10:00 P. M.	85	....	...	....	.....	....	....	....
" 9	9:15 A. M.	62	....	238	"	"	5.83	....	Not fed. Injected 6 cc. adrenalin in peritoneum.
	12:00 M.	156	....	...	....	.....	....	....	....
" 10		...	....	190	"	"	5 18	....	No adrenalin today. Not fed.
" 11	1:30 P. M.	88	44.7	68	"	"	1.82	....	Not fed. Injected 8 cc. adrenalin intraperitoneally.
	6:00 P. M.	86	....	...	....	.....	....	13	....
" 12	9:30 A. M.	54	41.9	100	"	"	1.25	....	No adrenalin today.

Total N output for 15 days	73.88
Average N output per day	4.92
N output per kg. per day	0.31

TABLE 7.

Dog F6-07.

Date 1917	Time	BLOOD			URINE					Body Weight kg.	Remarks
		Plasma Sugar mg. per 100 cc.	CO_2 vol. %	Corp. vol. %	Vol. cc	Sugar gm.	Acetone qual.	Total-N gm.	NH_3-N gm.		
Dec. 8	11:00	122	...	...	600	4 40	Neg.	8 88	0.33	14.5	Injected 6 cc. adrenalin into the peritoneum.
	1:00	345	....	....	...	....	...	.	...	....	
" 9		...	....	..	360	1 62	"	5 16	0.24	....	Injected 6 cc. adrenalin.
" 10	11:45	172	70.0	48 5	300	Faint	"	3 66	0.16	.	" 8 cc. "
	2:00	833	65.3	50.0	...	..	..	..	....	....	....
	5:00	192	59.5	47 2	...	..	...	....	....	....	....
" 11		...	....	....	400	Neg.	"	5 36	0 27	...	Injected 6 cc. adrenalin.
" 12		..	....	....	220	"	"	3.64	0.19	....	" " "
" 13	12:00	143	50.0	45 6	500	Faint	"	3.05	0 18	....	" " "
	2:00	322	59.5	43.3	...	....	....	....	....	....	....
	4:45	232	66.2	46.5	...	....	....	....	....	....	....
" 14		..	....	....	360	Neg.	"	5.52	0.30	11.25	No adrenalin. Hind legs spastic.
" 15		...	....	....	200	Mod.	"	5.26	0.26	....	Injected 8 cc. adrenalin.
" 16		178	63 3	42 3	145	Neg.	"	3.84	0.12	....	....

Total N output for 9 days	44.37
Average N output per day	4.93
N output per kg. per day	0.34

TAB. 8

Dog

Date 1917	Time	BLOOD			URINE					Body Weight kg.	Remarks
		Plasma Sugar mg per 100 cc.	CO₂ vol. %	Corp. vol. %	Vol. cc.	Sugar gm.	Acetone qual.	Total-N gm.	NH₃-N gm.		
Dec. 8	11:00 A. M.	179	....	....	640	Neg.	Neg.	10.36	0.59	15.0	....
" 9		...	....	...	240	"	"	3.87	0.20	....	....
" 10	2:00 P. M.	151	64.3	41.0	400	"	"	4.88	0.32	....	....
" 11		...	....	....	240	"	"	3.48	0.07	....	....
" 12		...	....	....	250	"	"	3.84	0.19	....	....
" 13	5:15 P. M.	93	62.4	41.2	300	"	"	5.46	0.30	...	....
" 14		...	....	....	200	"	"	4.84	0.16	12.2	0.5 gm. pancreatic tissue removed.
" 15		...	....	....	220	"	"	3.30	0.28	....	....
" 16		...	....	....	235	"	"	3.53	0.35	....	....
" 17		...	....	....	240	"	"	4.32	0.28	11.6	....
" 18	10:30 A. M.	105	61.4	....	280	Faint	"	4.17	0.18	....	....
" 19		...	....	....	250	"	"	4.05	0.22	11.0	1.25 gm. pancreatic tissue removed.
" 20		...	....	....	300	Neg.	"	3.72	0.33	....	
" 21		...	....	....	250	"	"	4.65	0.36	....	....
" 22	12:45 P. M.	100	....	....	280	"	"	3.90	0.33	....	....
" 23		...	....	....	100	"	"	1.24	0.26	. .	....
" 24	12.30 P. M.	130	52.8	31.9	200	"	"	4.10	0.19	....	Fed 100 gm. bacon grease.
" 25		...	....	....	120	"	"	1.82	0.13	....	" " " "
" 26		...	....	....	170	"	"	1.78	0.17	....	" " " "
" 27		...	....	....	230	"	"	3.63	....	....	" 50 gm. " " and 50 gm. lard.
" 28		...	....	....	150	"	"	1.82	0.40	....	
" 29		...	....	....	130	"	"	1.36	0.12	9.1	....
" 30		...	....	....	...	....	....	....	....	....	This 24 hr. urine lost.
" 31	5.00 P. M.	256	53.8	31.1	180	Neg.	Neg.	2.22	0.17	8.5	....

Total N output for 24 days	86.34
Average N output per day	3.59
N output per kg. per day	0.24

Though the familiar transitory adrenalin glycosuria is represented here, no definite influence toward the production of true diabetes is demonstrable, though mere over-feeding would have shown this effect very quickly. The rapid hypertrophy of the pancreas remnant in dog F6-08 may have more than compensated for its smaller size, and the slight difference in vacuolation of islands probably lies within accidental variations. Neither the glycosuria nor the azoturia of true diabetes was in evidence.

DOGS F6-06, F6-09, F6-10 AND F6-11.

These four animals were chosen as practically identical in size. All of them fasted completely from December 5 onward. On December 6, dog F6-06 was partially depancreatized, leaving a remnant about the main duct estimated at one-seventeenth, and dog F6-09 likewise, leaving a remnant estimated at one-fifteenth of the gland. Dog F6-06 was treated with adrenalin intraperitoneally, while dog F6-09 was used as a diabetic control. Dogs F6-10 and F6-11 were used as non-diabetic controls. The former received adrenalin intraperitoneally in the same dosage as dog F6-06. Dog F6-11 remained on plain fasting until December 19, when partial pancreatectomy was performed, leaving a remnant estimated at one-fifteenth, in order to compare the periods before and after this operation in the same animal. The initial body weight was used as a basis for reckoning the average nitrogen excretion per kilogram.

The output of sugar and nitrogen in dog F6-06 was by far the highest of the series, and the difference seems to lie outside the possible limits of accidental variation. The experiment indicates that, in the presence of active diabetes, adrenalin is able to cause a greatly increased excretion of sugar derived not merely from preformed carbohydrate but also from protein.

In the normal dog F6-10, adrenalin caused no glycosuria. The average nitrogen loss was higher than in the first ten-day period of dog F6-11, indicating a possible increase of nitrogen catabolism by adrenalin in a fasting animal, even without loss of sugar. This nitrogen loss, however, was lower than in the diabetic dog F6-09, which received no adrenalin but lost considerable sugar.

The results in dog F6-11 are uncertain. Owing to the prolonged fast, there was very little glycosuria. In figuring the nitrogen loss per kilogram, the initial weight of 19 kg. was taken as a basis for the first period and the weight of 14.55 kg. as a basis for the second period. The average loss thus appears higher during the second period, when the animal was potentially diabetic, but this basis of comparison may not be sufficiently accurate.

The pancreatic tissue of dog F6-06, taken in the original operation on December 6, was normal. The remnant at this operation was estimated at 1.78 gm. After further removal of 0.3 gm. on December 14 and 0.5 gm. on December 19, the remnant at autopsy weighed 2.2 gm. Microscopically, the islands showed vacuolation, increasing from a slight degree on December 14 to a moderate stage at autopsy.

TABLE 9.

Dog F6-06.

Date 1917	Time	BLOOD Plasma Sugar mg per 100 cc	BLOOD CO₂ vol %	BLOOD Corp vol %	URINE Vol cc	URINE Sugar gm	URINE Acetone qual	URINE Total-N gm	Body Weight kg.	Remarks
Dec. 8	11.00 A. M.	333	.		800	25 6	Neg.	12 48	19 0	Injected 6 cc. adrenalin intraperitoneally.
	1.00 P. M.	526		. .	. . .					....
" 9		. .			1500	55 5	"	9 40		Adrenalin the same as yesterday.
" 10	11.45 A. M	455	73 9	52 1	1350	44 1	"	14 14		Injected 8 cc. adrenalin in peritoneum.
	2:00 P M.	435	63 3	49 4						. . . .
	5:00 P. M	346	. .	48 2		.	.	.	. .	.. ..
" 11		.			900	Heavy	"	13 32		Injected 6 cc. adrenalin.
" 12		.	.		760	"	Slight	9 76		" " "
" 13	12:00 M.	500	57 6	51 5	900	28 71	Neg	16 29		" " "
	2:10 P. M	625	61 4	53 0			. . .	.	. .	. ...
	4:45 P. M	500	77 4	58 0		.	. .	.		. ...
" 14		.	. .	.	800	19 44	"	9 36		0 3 gm pancreatic tissue removed.
" 15				.	1000	28 3	"	11 84	. .	Injected 8 cc. adrenalin.
" 16		.			1400	32 2	V. faint	15 96	.	" " "
" 17				. .	900	25 2	"	10 53	11 95	" " "
" 18	10:30 A. M.	625	73 9	. . .	1000	30 9	Neg	13 76	.	" " "
	5:15 P. M.	625	60 3	. .		.	. . . .	. .	. . .	....
" 19		. .	. . .	. . . .	600	10 8	"	7 68	10 5	0 15 gm. pancreatic tissue removed
" 20	2:00 P. M.	525	55.7	42.2	640	13.72	"	6 72		Autopsy blood.

Total N output for 13 days		151 24
Average N output per day		11.63
N output per kg. per day		0.61
Total sugar output for 13 days	314 47	
Average sugar output per day	24.19	
Sugar output per kg. per day	1.27	

'ABLE 10.

Dog F6-09.

Date 1917	Time	BLOOD		URINE				Body Weight kg.	Remarks
		Plasma Sugar mg. per 100 cc.	CO_2 vol. %	Vol. cc.	Sugar gm.	Acetone qual.	Total-N gm.		
Dec. 8	11:00 A. M.	242	....	200	Neg.	Neg.	....	18.75	....
" 9		...	....	800	13.60	"	22.08	.....	....
" 10	2:00 P. M.	192	59.5	500	3.90	"	10.80	.....	....
" 11		...	....	360	Slight	"	6.48	.....	....
" 12		...	....	300	Neg.	"	6.45	.....	....
" 13	5:15 P. M.	312	60.5	370	"	"	8.56	.....	....
" 14		...	...	370	"	"	8.72	15.25	....
" 15		...	....	480	"	"	9 60	.....	....
" 16		...	....	405	14.50	"	9.75	.....	....
" 17		. .	....	460	18.50	"	10.75	13.85	....
" 18	10:30 A. M.	500	71 9	600	18 60	"	13.44	.....	....
" 19		...	....	400	13.80	"	8.52	12.6	0.6 gm. pancreatic tissue removed.
" 20		...	....	470	12.00	"	13.05	.....	....
" 21		...	....	740	6.16	"	15.52	.....	....
" 22	12:45 P. M.	147	....	450	Neg.	"	9.90	.....	....
	3:00 P. M.	79	71.0	...	....	.....	.....	11.5	....
Total N output for 15 days							153.62		
Average N output per day							10.24		
N output per kg. per day							0.54		
Total sugar output for 15 days					101 06				
Average sugar output per day					6.74				
Sugar output per kg. per day					0.36				

'ABLE

F6

Date 1917	Time	BLOOD			URINE					Body Weight kg.	Remarks
		Plasma Sugar mg per 100 cc.	CO_2 vol. %	Corp. vol. %	Vol. cc.	Sugar gm.	Acetone qual	Total-N gm.	NH_3-N gm.		
Dec. 8	11:00 A. M.	120	....	....	400	Neg.	Neg.	6.64	0 35	19 5	Injected 6 cc. adrenalin intraperitoneally.
	1:00 P. M.	222	....	....	...	"	"	....	....	....	....
" 9		...	....	....	870	"	"	3.87	0 25	....	....
" 10	2:00 P. M.	270	40.4	60 6	360	"	"	8 64	0 38	...	11 A. M. injected 8 cc. adrenalin.
	5:45 P. M.	132	61.4	65 0	...	"	"	....	...	....	....
" 11	10:45 A. M.	137	66 2	58 7	940	"	"	11 08	0 44	....	Injected 6 cc. adrenalin.
" 12		...	....	....	300	"	"	5 97	0 22	...	" " "
" 13	12:00 M.	70	72.9	60 5	340	"	"	9 12	0 48	....	" " "
	2:00 P. M.	294	64 3	57 6	...	....	...	....	....	....	....
	4:45 P. M.	70	56.7	61 5	...	...	....	....	...	....	....
" 14		...	....	....	300	"	"	7.50	0.45	14 7	No adrenalin today.
" 15		...	....	....	180	"	"	5 12	0 30	....	Injected 8 cc. adrenalin.
" 16		...	...	....	260	"	"	6 18	0.28	..	" " "
" 17		...	...	....	70	"	"	2.71	0 12	14 6	No adrenalin today.
" 18	10:30 A. M.	227	49.0	...	80	"	"	1 03	0 07	....	Injected 8 cc. adrenalin.
	5:15 P. M.	185	....	....	...	...	....	....	....	....	....
" 19		244	42.4	...	450	"	"	4 45	0 20	14.3	Autopsy blood.
Total N output for 12 days								72.31			
Average N output per day								6 02			
N output per kg. per day.								0 31			

Dog F6-11.

Date 1917	Time	BLOOD			URINE					Body Weight kg	Remarks
		Plasma Sugar mg. per 100 cc	CO₂ vol. %	Corp. vol. %	Vol cc.	Sugar gm.	Acetone qual.	Total N gm.	NH₃-N gm.		
Dec. 9		...	. ..	...	350	Neg.	Neg.	10.84	0 68	19 0	... .
" 10		...	....	....	100	"	"	2 97	0 13	....	.. ..
" 11		...	....	....	No Urine		.. .	.. .	....	....	.. ..
" 12		...	....	.	"	"	. .	.	....	....	.. ..
" 13	5:15 P. M.	82	54.8	48 2	220	Neg.	"	7 17	0.42	....	.. ..
" 14		...	. ..	....	230	"	"	3.81	0.33	15.8	.. ..
" 15		...	.. .	...	No Urine		.	....	. .	....	....
" 16		. .	....	.	"	"	. . .	. . .	. . .	....	....
" 17		...	...	. .	320	Neg	"	9 96	0 48	15.15	....
" 18		. .	....	...	170	"	"	3 36	0 12	....	....
" 19		...	.. .	. .	150	"	"	4 16	0 15	14 55	26.3 gm. pancreatic tissue removed.
" 20		...	....	. .	390	"	"	....	....	....	....
" 21		...	....	. ..	930	"	"	9 28	0 40	....	. ...
" 22		...	....	....	300	1 56	"	6 64	0 17	....	....
" 23		...	....	..	430	3 10	"	4 65	0.26	....	....
" 24		. .	. ..	.. .	700	Neg.	"	4 90	0.22	...	....
" 25		...	. .	.	720	"	"	4 32	0.25	....	....
" 26	9:30 A. M.	278	67 2	39 5	670	"	"	4 62	0 39	....	....
" 27	10:00 A. M.	357	..	.	840	"	"	6 57	0.86	....	0.1 gm. pancreatic tissue removed.
" 28		...		....	360	"	"	2.92	0 60	....	....
" 29		.		.	680	"	"	5 17	0 84	....	....
" 30		..		..	650	"	"	5 39	0.70	....	....
" 31	8:00 P. M.	1050	74 8	33 3	530	"	"	7.86	0 55	11.25	....

Total N output for 23 days	104 59
Average N output per day	4 55
N output per kg. per day	0 24
Total N output from Dec. 9 to 18	38.11
Average N output from Dec. 9 to 18	3.81
N output per kg. per day from Dec. 9 to 18	0.20
Total N output from Dec. 19 to 31	66 48
Average N output from Dec. 19 to 31	5.11
N output per kg. per day from Dec. 19 to 31	0 35

Dog F6-09 had a pancreas remnant estimated at 2.6 gm. on December 6. Additional tissue weighing 0.6 gm. was removed on December 19, and the remnant at autopsy weighed 1.7 gm. The tissue removed December 6 was normal. Distinct vacuolation was present in the islands on December 19, and at autopsy this was fully equal in degree to that found in dog F6-06. The Armanni changes in the renal tubules were similar in the two dogs No visible changes were therefore produced by the adrenalin doses given to one of these diabetic animals.

Dog F6-10 showed strictly normal pancreatic tissue at autopsy, with no sign of change due to the adrenalin injections. The kidneys showed congestion of glomuruli and slight degeneration and exudation in occasional glomuruli and tubules, which may or may not have been the result of the adrenalin. Armanni vacuolation was slight or uncertain, and the examination was not checked by glycogen or fat stains.

TABLE 13.

Dog F6-75 May 13, 1918.

Time	BLOOD			URINE			Remarks
	Plasma Sugar mg per 100 cc	CO_2 vol %	Corp vol %	Vol cc	Sugar gm	Acetone qual	
12:30 P. M.	132	46.2	41.8				10 cc adrenalin injected.
12:55 P. M.	232	51.9	50.0				
1:45 P. M.	555	46.2	49.0				
3:14 P. M.	558	50.0	50.6				
4:15 P. M.	445	61.4	56.7				
7:30 P. M.	99	54.8	68.5				
9:30 P. M.	112	51.9	68.0	92	3.32	Neg.	Urine for experimental period.

Dog F6-11 showed normal pancreatic tissue in the first operation on December 19, except for partial emptiness of the acini due to fasting. No alteration of islands due to fasting, and none of the alleged transitions between acinar and island tissue were discoverable. Slight vacuolation was found in the islands on December 27, and a slightly more advanced stage at autopsy on December 31. This vacuolation is evidently due to the degree of functional overstrain represented by the marked hyperglycemia, as glycosuria was absent most of the time.

DOG F6-75.

This normal animal, weighing 7.4 kg., was given 10 cc. of adrenalin solution intraperitoneally at 12:30 P. M. Great hyperglycemia resulted, but the total sugar excretion was only 3.32 gm. The animal was found dying shortly after midnight, and was autopsied immediately. The pancreas was strictly normal, and neither acini nor islands showed the slightest microscopic changes.

DISCUSSION.

I. Alleged Antagonism of Epinephrin and Insulin.

With the recent discovery of insulin, the last requirement for complete demonstration of the pancreatic origin of diabetes has been fulfilled. What remains of opposition to this doctrine consists not of facts, but merely a state of mind. The development of the truth has been so slow that a multitude of persons have become accustomed to pluriglandular conceptions of diabetes, and the most conclusive proof will encounter difficulty in uprooting an error which is so deeply grounded in the literature. There is danger that the same shallow thinking which founded the pluriglandular doctrine will introduce further confusion by the assumption that epinephrin is the physiological antagonist of insulin. Epinephrin causes glycogenolysis and hyperglycemia; insulin causes glycogen formation and lowering of the blood sugar. What could be a plainer example of antagonism?

As an anatomical example of a similar fallacy, it may be suggested that the chin is lowered when the mouth is opened or the head bent forward, and elevated when a man rises from a sitting posture or leaps into the air; and a person confining his attention exclusively to the chin might thus conclude that the depressors of the mandible are the natural antagonists of the extensors of the legs. If epinephrin is the physiological antagonist of insulin, then the chromaffin tissue is the opponent of the islands of Langerhans, which means that the doctrine of antagonisms and balances between glands is correct, and that diabetes is a form of epinephrin glycosuria, due wholly or largely to the unrestrained action of the adrenals when the inhibiting influence of the Langerhans tissue is weakened. A presentation of experimental evidence on the subject is therefore important at this particular time. Pluriglandism is to be opposed not merely as a false doctrine but especially as an unscientific mode of thought which constitutes the most pernicious tendency in the contemporary study of metabolism. It is therefore worth while to recapitulate briefly some of the known facts bearing on the relation of the adrenals to glycosuria or diabetes, under the following five heads:

1. Clinical Pathology.—It is bad enough that rare abnormalities of the thyroid or hypophysis in association with diabetes have caused these to be dragged into the etiology, but it is well known that for the adrenals no trace of evidence for such a connection exists. The signs of adrenal excess consist in virilism, hirsutism, etc.,[33,34,35] and are supposedly due to overdevelopment of the cortical tissue. No state of chromaffin excess has ever been recognized. The numerous attempts to demonstrate abnormal quantities of epinephrin in the blood or in the adrenals have failed completely in diabetes, hypertension, and all other conditions.[36,37] Various grades of diabetes and hypertension coexist in numerous elderly patients, doubtless on the basis of associated scleroses in the pancreas and kidneys; but believers in an epinephrin factor have never been able to explain why hypertension is so often found with normal carbohydrate tolerance and why the great mass of diabetics have normal blood pressure.[38] Likewise, states of chromaffin deficiency have never been demonstrated, and there is no evidence that the hypoglycemia of Addison's disease is due to lack of epinephrin.[16]

2. Epinephrectomy Experiments.—It is impossible to learn whether the chromaffin tissue is essential to life, because the greater part of this tissue lies outside of the adrenals and cannot be removed. The cortical tissue, which does not produce epinephrin, is essential to life, and its removal is responsible for the acute death which follows double epinephrectomy in most species. A large proportion of animals of some species, such as the rat and the rabbit, survive this operation because they possess sufficient cortical tissue outside the adrenals.[1,16,18] Species such as the cat and dog, in which the double operation is invariably fatal because of lack of sufficient accessory cortical nodules, survive indefinitely when the medullary substance is removed and the cortex spared.[17,21] Animals which have thus lost the entire adrenal medulla show nothing resembling Addison's disease, have normal blood sugar and carbohydrate metabolism, and are subject to diabetes and various forms of experimental glycosuria in the usual way.[20] The hypoglycemia and other fatal symptoms are due to loss of the cortical tissue, and both the experiments and the interpretations underlying the pluriglandular hypothesis are thus proved to be erroneous.

3. Epinephrin Injections.—The first step in an attempt to show a relationship of the chromaffin tissue to diabetes should be a proof that epinephrin can cause glycosuria. As previously pointed out,[39] such proof has never been brought. The most obvious and characteristic effect of epinephrin in the living organism is the elevation of blood pressure. It remains a fact that the method of administering epinephrin which produces this typical effect to the highest degree, namely intravenous injection, has the least influence upon carbohydrate metabolism, and, *vice versa*, subcutaneous or intraperitoneal injections which cause little or no rise of blood pressure produce the greatest hyperglycemia and glycosuria. Attempted explanations of this discrepancy are unsatisfactory, and it is a permissible hypothesis that the excess sugar formation is due to chemical products from epinephrin decomposition or from the injured cells, such secondary changes occurring to some extent when the epinephrin is given intravenously but to a greater extent when it is injected directly into the tissues. Epinephrin is supposed, particularly by the pluriglandists, to exert its glycogenolytic influence chiefly in the liver. The anatomic situation of the adrenals is such that their epinephrin discharge is equivalent to an intravenous injection in the systemic, not the portal, domain, and this arrangement therefore seems in all respects least adapted to act upon the liver glycogen. It is therefore possible that the hyperglycemia and glycosuria from epinephrin injections represent a purely artificial drug effect, not corresponding to anything in either normal or pathological metabolism. The argument that any marked pharmacologic property of a glandular extract must indicate a similar activity on the part of the living gland is best answered by the example given by Kennaway and Mottram,[40] namely, the galactagogue substance which is found in the pituitary glands of fish. If all the above objections be discarded, the question of dosage is still too important to be ignored. The quantities of epinephrin used to produce glycosuria are huge in a physiological sense. It is doubtful if any such quantities are thrown into the circulation from the adrenals under either normal or abnormal conditions, and it is certain that if such quantities are ever poured into the veins they must cause elevated blood pressure and other disturbances. No such thing as a prolonged continuous epinephrin glycosuria has ever been produced, and the idea is absurd on the face of it, because of the renal injuries and systemic intoxication which must result from the large quantities required for this purpose in either normal or potentially diabetic animals. On the other hand, small doses of epinephrin lower the blood pressure instead of raising it,[41,42,43,44] and likewise lower the blood sugar instead of raising it.[45] Repeated small doses of epinephrin increase glycogen storage.[46] These interesting results of small quantities, which perhaps fall within the limits of physiological epinephrin production, have been completely overlooked in the current speculations on the subject.

4. *Function of the Chromaffin Tissue and Epinephrin.*—Several facts are opposed to a specific antagonism between epinephrin and insulin. First is the different action of different quantities of epinephrin just mentioned. Second, if it should be granted that the acute disturbance following double epinephrectomy is in any way due to deficiency of epinephrin, it could still be pointed out that the pluriglandists as usual have seized upon the one feature which suits their views, namely, the hypoglycemia, and have ignored the other equally striking occurrence, namely, the rapid disappearance of glycogen and loss of power to form glycogen,[47,48] which is the direct opposite of the results of insulin excess. Third, the effect of epinephrin as an antidote to an overdose of insulin, which seems so striking, represents merely sudden glycogenolysis, entirely comparable to such an artificial procedure as a glucose injection. It is not only susceptible to interpretation as an unphysiologic drug action, as above mentioned, but also is apparently dependent upon the presence of glycogen. Epinephrin probably cannot antidote an overdose of insulin in a diabetic who lacks sufficient glycogen.[49] Absence of insulin results in glycosuria which is not dependent upon glycogen but continues at the expense of maximum sugar formation from protein. This fact alone warrants the inference that epinephrin is not a specific antagonist of insulin, and that deficiency of the one hormone is not equivalent to excess of the other. From a broader viewpoint, attention may be directed to the results of all the recent investigations of the function of the chromaffin tissue and epinephrin. The views of Cannon and Stewart, opposed in several particulars, are fully harmonious on this point. Stewart's criticisms have exposed

the speculative and erroneous opinions which have been so widely entertained concerning the role of the adrenals, and his researches and those of his pupils[50 to 66] have tended to discredit epinephrin excess as an explanation of various glycosurias and other experimental phenomena, and thus to shatter the entire pluriglandular conception of this question. Cannon[67,68,69,70] and others[71,72,73,74] have championed the emergency function of the chromaffin tissue, in the sense that a discharge of epinephrin under a strong nervous stimulus increases muscular tone and also mobilizes extra sugar as fuel for strenuous activity. This provision might be highly advantageous; but, with recognition of the fact that diabetes means inability either to burn or to store carbohydrate, it is at once evident that any tendency to diabetes would be extremely disadvantageous under these circumstances and would defeat the entire purpose of the mechanism. The entire embryology and phylogeny of the chromaffin system indicate some functional association with the sympathetic nervous system, which is something altogether apart from diabetes.

5. Nature of Diabetes.—The basic characteristic of diabetes is the inability to utilize food, particularly carbohydrate. An overproduction of sugar may be a usual accompaniment, but clear thinking must classify a depancreatized animal as diabetic to the same degree as before, even if hypoglycemia has been produced by removal of the adrenals or liver or by any profound shock or exhaustion, because insulin and the normal nutritive function which depends upon it are still absent. That epinephrin does not prevent sugar utilization is proved by the retention of a large proportion of any dosage of sugar even at the height of epinephrin glycosuria,[1,29] and by respiratory studies,[75 to 79] particularly those of Lusk.[80,81] Most persons who lightly accept an antagonism between epinephrin and insulin fail to recognize that this is one of four doctrines of the Vienna school which are inseparably bound together. First, diabetes is supposed to be a pure overproduction of sugar, without any impairment of utilization; the glycosuria merely conforms to Pflüger's idea of "a glass running over." Second, the liver is depicted as the "sugar factory," in which the machinery of production is driven by epinephrin, while the function of insulin is merely to act as a "brake" to hold this production within normal limits.[82,83] Third, the doctrine of sugar formation from fat is an essential feature, because the alleged quantities of sugar, far in excess of anything derivable from carbohydrate or protein, can come from no other source. Fourth, the above views require denial of the generally accepted explanation of acidosis; therefore it is asserted that the acetone bodies are by-products of the formation of sugar from fat in the liver. These four Vienna doctrines, however unproved and pernicious, are at least consistent. Anyone, however, who accepts scientific proofs must recognize that the excess of sugar in diabetes is an expression of the profound nutritive disturbance. The function of insulin is not merely to build up glycogen or prevent sugar formation, but to provide for the normal utilization of carbohydrate and other foods and for the entire bodily nutrition. This specific nutritive function of insulin, and the reinforcement of sympathetic nervous activities by epinephrin, are processes of a totally different order. Glucose injections, which cause hyperglycemia, are not antagonistic to insulin, but on the contrary are utilized by means of insulin. Likewise epinephrin, piqûre, ether and all other agencies which cause hyperglycemia by glycogenolysis cannot be regarded as antagonistic to insulin, unless they tend to create true diabetes by abolishing the utilization of sugar. In the same sense, it is ridiculous to regard liver removal, shock, poisoning by peptone or hydrazine, or other causes of hypoglycemia as adjuvants to insulin or antagonists of epinephrin. The whole question comes down to the usual fallacy of the confusion between the disease, diabetes, and the mere symptom of glycosuria or hyperglycemia. It is like reverting to a stage of medicine in which all forms of fever are regarded as identical, and every means of reducing body temperature is supposed to counteract the cause of the fever. As long as the prostration of sugar utilization and of the entire normal nutrition stands as the essential characteristic of diabetes, no agent which merely causes glycogenolysis or hyperglycemia can properly rank as a physiological antagonist of insulin.

II. Abnormal Lability of Diabetic Tissues.

The fact which leads deepest into the understanding of diabetes is the influence of undernutrition and overnutrition. The insulin consumption of the body is affected by the total calories of the diet and by the body weight.[84] The latter factor is the more surprising, but it is established beyond mistake that obesity conduces to bring on active diabetes and that patients and animals tolerate higher diets and require smaller insulin dosage at low than at high body weights. Totally depancreatized animals not only suffer a rapid progressive breakdown of their tissues, but also show a lack of power to heal wounds or resist infection which is not explainable by simple glycosuria, hyperglycemia or cachexia. This condition is probably analogous to the well-known vulnerability of the tissues of patients with active diabetes, as manifested by numerous infectious complications, such as carbuncle and gangrene, and by the non-infectious complications, such as cataract, retinitis, arteriosclerosis, etc. Control of the diabetes by diet, even when this involves undernutrition to the point of cachexia, confers practically absolute immunity against all complications.[85] Therefore, in some way, the tissues are healthier with the low nutrition of inanition than with the specific malnutrition of active diabetes. The writer formerly[86] brought evidence that the Langerhans hormone is concerned not only in catabolism but also in anabolism and the upbuilding and maintenance of the entire body mass. Though the storage of insulin in the body must be small, as proved by the quick onset of diabetes after pancreatectomy, there must be some explanation of the remarkable fact that an organism at a very high level of weight seems to use up actually several times as much insulin as the same organism at a very low level of weight.[84] A corollary of the above hypothesis is that deficiency of insulin must create difficulty in building up or repairing tissue and a tendency to breakdown of existing tissue. The signs of impairment of resistance and nutrition in all parts of the diabetic body are thus easily explained. Tests of this hypothesis cannot be made with substances which introduce an element of cachexia or otherwise interfere with sugar formation, but they are to some extent possible with substances which tend to increase sugar formation. Such tests may give some indication whether the living protoplasm of the diabetic organism is more readily broken down into sugar than that of the non-diabetic organism.

The experiments with epinephrin in normal dogs, in partially depancreatized dogs without active diabetes, and in partially depancreatized dogs with active diabetes, showed the following results:

(a) In the normal animals epinephrin produced the familiar hyperglycemia and glycosuria, derived evidently from the glycogen stores as commonly understood, and with no indication of direct sugar formation from protein. The total sugar excretion was small, and the assertion was ventured that no form of administration can be devised which can maintain heavy continuous glycosuria, because of the fatal intoxication which must quickly result from the doses of epinephrin required for this purpose.

(b) The only possible contention of the upholders of glandular antagonisms must be that with deficiency of insulin the influence of epinephrin upon sugar production is exaggerated, so that heavy continuous glycosuria may be kept up, even at the expense of protein decomposition, by quantities of epinephrin which do not cause elevation of blood pressure or toxic symptoms in the typical diabetic. Dogs D4-77 and E5-00 were severely diabetic, as proved by the low diets required to bring their diabetes under control. Dog F6-07 had only one-eleventh of the pancreas, but sugar freedom was maintained by fasting before and after the operation. Dog D4-77 received slow intravenous injections of epinephrin in dosage which produced obvious effects upon the cardiac action and blood pressure, though no record of these was kept. No glycosuria resulted. All these dogs received epinephrin by the methods which are most effectual for glycosuria, namely, subcutaneous or intraperitoneal injections, but the glycosuria was slight and transitory, with no evidences of progressive tendencies or overproduction of sugar from protein. These experiments seem to be fairly crucial regarding the pluriglandular hypothesis, because if epinephrin is a true antagonist of insulin it should produce some tendency to diabetes under these conditions. If it places the Langerhans tissue under strain, the few remaining islands should show hydropic changes. If it in any way inhibits or paralyzes the action of insulin, marked diabetic symptoms should result with such a scanty insulin supply. If it can reproduce the chief characteristic of diabetic glycosuria, namely the excretion of sugar derived from protein, this power should be plainly manifest when the alleged "brake" action of insulin is thus weakened. The negative results under all these heads appear conclusive.

(c) Absence of glycosuria may be taken as an indication that the organism possesses at least the minimum amount of insulin necessary to prevent the abnormal breakdown of its tissues to form sugar. Correspondingly, diabetics free from glycosuria are ordinarily immune to complications. Therefore a demonstration of an abnormal lability of diabetic tissues can be anticipated only with a deficiency of insulin sufficient to cause active symptoms. In the actively diabetic dog F6-06, epinephrin injections produced a great increase of both sugar and nitrogen excretion, as compared with the various controls; namely, F6-08 and F6-11, fasting normal or diabetic animals without active symptoms; F6-10, a fasting normal dog receiving adrenalin injections; F6-07, a fasting diabetic dog without active symptoms, receiving adrenalin injections; and F6-09, a fasting diabetic dog with active symptoms without adrenalin. The increase of nitrogen in dog F6-06 is specially important as showing that the extra glycosuria represented not merely a sweeping out of preformed carbohydrate but an actual increase of protein destruction extending over a number of days. Eppinger, Falta and Rudinger reported an increase of glycosuria from adrenalin in a totally depancreatized dog. A choice may be necessary between two explanations, namely the pluriglandular view of an epinephrin element in diabetes, and the hypothesis of an abnormal lability of diabetic tissues. Regarding the first, it must be recognized that the symptoms of existing diabetes may be aggravated by many agencies (carbohydrate excess, thyroid intoxication, systemic infections, etc.) which have not the slightest influence as primary causes of diabetes. Also, it is believed that the previous experiments and discussion sufficed to eliminate epinephrin as a direct antagonist of insulin or a diabetogenic agent.

In any event, the observations show that epinephrin injections caused an excess elimination of sugar and nitrogen in an animal with active diabetes, out of all proportion to the results in normal or potentially diabetic controls. Such experiments are open to different interpretations, and do not prove unequivocally that the tissues with active diabetes are abnormally subject to breakdown. Owing to the incidental difficulties, several other attempts of this kind failed and only this one series was carried through satisfactorily, so that confirmation in a larger number of animals is desirable. The results obtained, however, at least harmonize well with the other evidence of the role of insulin in upbuilding and maintenance of the body and the abnormal lability of tissues arising from the lack of insulin.

CONCLUSIONS.

1. Removal of most of the adrenal tissue of dogs did not alter the carbohydrate tolerance as judged by intravenous injections of glucose. The tendency to diabetes was also unaffected, apart from the influence to be expected from any fatal cachexia.
2. Fat feeding created no increased tendency to epinephrin glycosuria. A resemblance to diabetic glycosuria in this respect was therefore not demonstrable.
3. Epinephrin injections over a series of days failed to produce active diabetes in dogs which already had severe latent diabetes in consequence of extensive pancreatectomy. The glycosuria was only a trifle greater than in normal animals with the same dosage, and was evidently due as usual to glycogen breakdown, as no indications of overproduction of sugar from protein were found. Tendencies to progressiveness, or signs of functional strain in the form of vacuolation of the remaining Langerhans island cells, were also not produced by epinephrin.
4. The above evidence, together with much already existing in the literature, is interpreted as proving that epinephrin is not a diabetogenic agent or a physiological antagonist of insulin.
5. In an animal with active diabetes, epinephrin injections caused a greatly increased excretion of both sugar and nitrogen, as compared with normal or diabetic controls. The most important feature was the extra destruction of protein continuing over a number of days, indicating that the increased glycosuria was not a mere sweeping out of preformed carbohydrate. No positive interpretation is established, but this result harmonizes with other evidence of an anabolic role of insulin, which is essential for upbuilding and maintaining the entire body mass, and of an abnormal susceptibility to breakdown of the tissues with active diabetes.

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EXPERIMENTAL STUDIES IN DIABETES.

SERIES II. THE INTERNAL PANCREATIC FUNCTION IN RELATION TO BODY MASS AND METABOLISM.

12. DIABETES AND PHLORIZIN GLYCOSURIA.

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The use of phlorizin is classical, both for experiments concerning metabolism as such, and in attempts to imitate the conditions of true diabetes. It was tried for the present purpose in connection with three questions: (1) the special lability of diabetic tissues; (2) the influence of phlorizin glycosuria upon diabetes; and (3) the comparative effects of phlorizin glycosuria and diabetes.

1. Special Lability of Diabetic Tissues.

The question here is the same as in the preceding paper,[1] namely whether it is possible to demonstrate a greater tendency to breakdown in diabetic tissues than in normal tissues under the same conditions. It is known from the early literature[2] and from general experience that the glycosuria from a given quantity of phlorizin differs somewhat with the ready availability of glucose. The same dose, for example, causes greater sugar excretion in an animal on abundant protein diet than on fasting, and still greater on high carbohydrate diet. If, therefore, the tissues break down into sugar more readily in the diabetic than in the normal organism, there is a chance that this difference might be revealed by phlorizin.

One step consisted in comparisons between normal dogs and partially depancreatized dogs free from glycosuria. These were placed either on fasting or on identical diets, and then given single subcutaneous injections of either 1 gm. or 0.5 gm. of phlorizin suspended in oil. Comparisons were made of the subsequent excretion of both sugar and nitrogen. The reproduction of several dozen detailed records may be avoided by the brief statement that no significant differences were found. Irrespective whether dogs have mild or severe diabetes, if their sugar has been thoroughly controlled by diet or fasting, they react to phlorizin in the same way as normal dogs subjected to the same conditions of nutrition. In other words, no increased lability of the body protein of diabetic animals is demonstrable by this method when the diabetes is under control by diet.

The results are different when active diabetic symptoms are present. The higher sugar excretion, as compared with normal dogs, is then so obvious that again most of the records may be omitted.* One example has already been published in another connection.[4] It was there shown that in dog F6-02 the D:N ratios were markedly higher after removal of enough pancreatic tissue to produce diabetes, even though the animal was fed before the operation and fasting after it. A mere sweeping out of preexisting carbohydrate is excluded by the corresponding increase of nitrogen which ordinarily occurs, indicating an acceleration of protein breakdown. Dog D4-60 (Table 1, below) may serve as a sufficient illustration of the differences of sugar and nitrogen output in the same animal according to whether diabetic symptoms were present or absent. Dog D4-49 (Table 2, below) received phlorizin only when the diabetes had led to a practically moribund state, and, though preformed glucose was actively eliminated as indicated by the fall of blood sugar, the usual increase of both sugar and nitrogen excretion was prevented by the cachexia.

These findings are summarized in this cursory manner, partly because they are fairly self-evident, but chiefly because they are inconclusive as proof of the point under consideration. Excessive excretion of sugar and nitrogen produced by phlorizin in diabetic dogs previously free from glycosuria would be strong evidence of an increased lability of body protein in such animals. The positive results in actively diabetic animals are less striking, because they may be interpreted as the mere superposition of two agencies, namely diabetes and phlorizin poisoning, both of which tend to cause glycosuria and azoturia. It can only be said that the results resemble those with epinephrin,[1] and correspond to theoretical reasoning. When the diabetes is under thorough dietary control so that glycosuria and hyperglycemia are absent, it is rational to suppose that the insulin supply is adequate to prevent any abnormal tissue breakdown. On the other hand, when deficiency of insulin is indicated by active diabetic symptoms, the excessive sugar and nitrogen loss resulting from phlorizin do not demonstrate an abnormal tissue lability but are at least in harmony with such an hypothesis.

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* The chief disturbances which may cause irregular results in such experiments are nephritis and cachexia. The reduction of phlorizin glycosuria by renal disease is well known. Phlorizin still causes glycosuria in the most extreme states of prostration;[3] nevertheless, the quantitative output is less with cachexia than with normal nutrition.

2. *Influence of Phlorizin Glycosuria upon Diabetes.*

Three facts must be considered in relation to this point.

(a) Phlorizin has no direct influence upon the islands of Langerhans. It does not cause hydropic changes, or prevent such changes when they are in progress.[4] This is merely one of many proofs that phlorizin poisoning is a totally different condition from true diabetes, so that confusion should not be created by applying the name diabetes to it.

(b) It is currently accepted that moderate doses of phlorizin cause no increase of either nitrogenous or total metabolism in animals receiving adequate rations of protein or carbohydrate. In fasting, however, the sugar which is not supplied by food must be derived from the tissues, and a tremendous increase in the nitrogen excretion and the respiratory exchange results.[5] As one purpose in this series of papers is to study the relation between total metabolism and diabetes, this behavior of phlorizin offers one opportunity.

(c) The sugar excreted in diabetes is completely lost to the organism in every sense. It is not burned, and has no influence on protein catabolism, acidosis or any other known phase of bodily chemistry. One of the striking differences between phlorizin glycosuria and diabetes is shown by the behavior of sugar, which escapes combustion but nevertheless affects the metabolism. Glucose spares protein in phlorizinized animals, even though the dose administered is quantitatively excreted.[6] Likewise, the ketonuria of fasting phlorizinized dogs is nearly or completely abolished by protein feeding, even when the entire theoretical glucose content of the protein is lost in the urine.[7] A special problem is thus created in relation to diabetes. Suppose that a diabetic dog is placed on a diet which produces glycosuria and which would therefore cause inevitable downward progress in the sense of progressive loss of tolerance; but at the same time the animal is given sufficient phlorizin to cause even greater glycosuria than resulted from the diabetes alone. The mere absence of hyperglycemia is not a decisive factor, as shown by the experiments already quoted.[4] The glucose (whether from starch or protein) which is in excess of the animal's tolerance must still be digested and carried through the blood, and it may enter into metabolism in some way indicated by the reduction of protein catabolism and acidosis. Do these processes constitute a functional demand upon the islands of Langerhans, so that the diabetes will still progress? Or does the specific functional demand consist only in the combustion of the sugar, so that phlorizin, by robbing the organism of more sugar than the excess contained in the diet, may actually furnish a means for taking the excessive diet without impairment of tolerance? A similar problem is presented in regard to the facts stated under (b). The nitrogen output and total metabolism of the fasting animal are greatly increased by phlorizin; but at the same time the sugar formed is excreted unburned and the increased metabolism accelerates the course of undernutrition. Which of the seemingly opposed processes actually prevails? Will the increased metabolism break down tolerance, or will the accelerated undernutrition build it up?

The first trials were made in fasting animals. In order to obtain any decisive proof of benefit from phlorizin, the attempt was made to choose dogs with diabetes of such severity that fasting barely failed to arrest it. In the testing of any large number of partially depancreatized dogs, it is not uncommon to find them at such a stage that the glycosuria falls to very small quantities after a few days of fasting, but then ceases to fall further or actually increases with prolongation of the fast. According to that view of diabetes which pays attention only to glucose, it should be a decided benefit to a diabetic animal to have all the glucose withdrawn from its fasting metabolism by phlorizin, so that it is forced to live entirely on non-carbohydrate materials. Such a benefit, however, is either absent or too slight to be demonstrated by the method chosen. It was never possible to show that a diabetes could be cleared up by fasting plus phlorizin when it was resistent to fasting alone.

On the other hand, experiments designed to test the harmlessness of phlorizin easily gave positive results. There is no doubt that animals, whose diabetes is controllable by fasting, respond equally well to the combination of phlorizin and fasting. Some impression was gained that this combination was more beneficial than plain fasting, in arresting the diabetes more rapidly or building up a higher tolerance. Here also definite proof of the benefit cannot be furnished, because no two animals are strictly identical and the diabetes in a single animal is not the same in tests performed at different times; also the tolerance can be judged only after the glycosuria from the phlorizin has ceased. It can, therefore, only be stated positively that the increased metabolism of fasting phlorizinized animals does not interfere with the control of the diabetes. This is an important practical point for the conduct of experiments, such as previously mentioned,[4] to show that active diabetes still causes hydropic degeneration of islands when the blood sugar is kept at a low level by phlorizin. The diabetes must be sufficiently severe to resist the undernutrition entailed by the phlorizin program (with or without fasting), or else the arrest of the active diabetes will naturally arrest the hydropic changes.

The effects of diet with phlorizin can be more definitely shown by protocols, and sample records will therefore be given, as follows:

Dog B2-62. The operative history of this animal has been previously published.[8] On May 22, 1914, approximately two-thirds of the pancreas was removed, and subsequent glucose tests by stomach and subcutaneously showed a tolerance of 8 gm. per kg., slight glycosuria resulting from 9 gm. per kg. Sept. 9, phlorizin was begun in dosage of 0.5 gm. subcutaneously. The dosage was gradually increased as high as 2 gm., sometimes given every day, and sometimes several days apart, in the attempt to maintain the highest possible glycosuria. The sugar excretion remained between 100 and 200 gm. per day. The last phlorizin injection was given on Nov. 18, and glycosuria ceased on Nov. 22. The dog had remained in excellent condition on an unrestricted diet of bread and soup, and the weight of 10.2 kg. was precisely the same at the beginning and end of the experiment. Nov. 23, a test with 9 gm. glucose per kg. showed the same tolerance as before the phlorizin period. Nov. 24, a specimen of pancreas weighing 0.2 gm. was obtained by operation, and microscopically was found normal in islands and acini. The previous report[4] of absence of influence of phlorizin upon the islands of Langerhans is supplemented by this observation of unchanged tolerance and unchanged histologic appearances.

Dog B2-63 was also mentioned in a former publication.[9] Operation on May 22, 1914, left a remnant estimated at 1/6 to 1/7 of the pancreas. For a time glycosuria could be maintained by bread diet with addition of 300 or 400 gm. of glucose daily, but by Aug. 3 the dog refused glucose, glycosuria ceased, and the attempt to break down the tolerance by diet had definitely failed. The diet was then changed to bread and soup without glucose, and phlorizin was begun in dosage of 1 or 2 gm., sometimes daily and sometimes with a day or two between injections. Glycosuria of 100 to 200 gm. daily was thus maintained. Pancreatic tissue weighing 1.32 gm. was removed by operation on the afternoon of Nov. 6, after an injection of 2 gm. of phlorizin that morning. The last phlorizin injection was given on Nov. 14, and glycosuria ceased on Nov. 18. The dog remained in excellent condition and gained weight slightly, from 23.5 kg. at the beginning of the phlorizin period to 23.9 kg. at its close.

The further record is given in the previous publication.[9] The dog's condition was one of extremely mild but permanent diabetes, which slowly became more marked during several months of subsequent carbohydrate feeding. This result is abundantly explained by the amount of pancreatic tissue removed. An even greater effect might have been anticipated had the operation of Nov. 6 been performed before the phlorizin period; therefore, a slight gain of tolerance during this period may be suspected. The tissue removed on Nov. 6 was normal in acini and islands, again illustrating the absence of effect of phlorizin upon the pancreas.

The conditions of this experiment required the dog to eat enough bread to maintain his nutrition and to supply in addition an excretion of 100 to 200 gm. of glucose daily in the urine. The question was whether the extra carbohydrate ingestion under these circumstances would overload the insular function in an animal so close to the verge of diabetes. The answer was in the negative.

Dog D4-60, a large male Irish terrier mongrel, in medium condition at a weight of 19.3 kg., was partially depancreatized on Dec. 14, 1916. The tissue removed weighed 42.5 gm., and the remnant left about the main duct was estimated at 3.7 gm. (1/12—1/13). The resulting severe diabetes was kept under partial control by diet, while tests showed that 500 gm. of beef-lung sufficed to cause glycosuria, and the feeding of 800 gm. caused glycosuria of about 20 gm. daily for 3 days. Beginning Dec. 21, phlorizin was administered in occasional small doses as shown in Table 1. The dog was not catheterized, but the irregularities in the daily urinary analyses due to irregular voiding do not interfere with the purpose of the experiment.

TABLE 1.

Dog D4-60.

Comparison of two periods on identical diet and phlorizin dosage.

Date	URINE					BLOOD PLASMA			Body Weight kg	Phlorizin gm.	DIET
	Vol. c.c.	Sugar gm.	T. N. gm.	D:N ratio	Acetone gm	Sugar mg per 100 c.c.	ACETONE				
1916							Qual	Total mg per 100 c c			
Dec. 21	961	21.1	...		Neg.	361	...	...	...	0.5	800 gm. lung.
" 21	1542	70.9	...	..	"	57	...	.....	18.1	..	" " "
" 23	1287	50.2	...	...	"	.	....	...	...	0.75	" " "
" 24	1409	83.1				.	....	..	...	....	" " "
" 25	804	50.7	.		Faint		..	....	.....	0.5	" " "
" 26	728	34.9	....	.	Slight	..	.....	...	......	0.5	1 kg. lung.
" 27	764	66.5	18.8	3.52	0.17	81	Neg.	4.0	17.1	.....	" " "
" 28	1280	83.2	30.0	3.04	0.40	....	....	.....	......	0.5	" " "
" 29	1395	75.2	28.5	2.64	0.22	.....	......	....	......	......	" " "
" 30	872	54.9	15.2	3.59	0.15	82	Neg.	.....	......	......	500 gm. lung, 100 gm. suet.
" 31	991	83.2	25.5	3.25	0.27	.....	....	......	......	0.5	" " " " " "
Jan. 1	408	31.4	11.6	2.72	0.29	....	....	.....	.....	......	" " " " " "
" 2	309	5.2	1.7	3.08	.	185	Faint	14.9	15.4	......	" " " " " "
" 3	1110	72.2	28.1	2.56	0.52	....	......	......	......	......	" " " 200 " "
" 4	550	34.8	13.8	2.58	0.52	73	V. Faint	......	......	......	" " " " " "
" 5	850	61.1	17.4	3.36	0.34	......	....	......	......	......	" " " " " "
" 6	542	21.1	6.2	3.32	0.21	.....	....	......	......	......	" " " " " "
" 7	1029	42.2	17.0	2.48	1.02	......	....	......	......	......	" " " " " "
" 8	544	30.5	10.4	2.92	0.37	49	Faint	......	......	......	" " " " " "
" 9	1055	56.9	8.8	....	0.25	57	....	......	14.9	0.5	" " " " " "
" 10	1865	121.2	14.9	....	......	......	....	......	......	......	" " " " " "
" 11	1295	54.0	10.8	....	0.28	58	....	......	14.5	......	" " " " " "

TABLE 2.

Dog D4-60

Date.	URINE					BLOOD PLASMA			Body Weight kg.	Phlorizin gm.	DIET
	Vol. c c.	Sugar gm	T N gm.	D N ratio	Acetone gm	Sugar mg per 100 c c	ACETONE				
							Qual	Total mg per 100 c.c.			
Feb. 7	684	Neg.	...	...	Neg.	...	...	...	15 2	0 5	800 gm. lung.
" 8	792	48.8	...	...	"	...		...	14 6	...	" " "
" 9	1255	24 2	...	...	"	...	...	...	14 9	0 75	" " "
" 10	1120	58.8	...	...	"	...	...		14 9	...	" " "
" 11	340	20 9	...	...	V. Faint	...	...		...	...	" " "
" 12	1024	62.9	...	...	Neg.	98	Faint	16 8	14 2	0 5	1 kg. lung.
" 13	866	47.7	21 4	2 23	Faint		...	...	14 5	0 5	" " "
" 14	988	47.6	23.6	2.02	Slight	...	...	...	14 1	...	" " "
" 15	1175	33 9	18 5	1 84	Neg.	...	...	...	14 0	...	" " "
" 16	621	31.7	13 0	2 44	"	...	...		13 9	...	500 gm. lung, 100 gm. suet.
" 17	212	Neg.	5 9		"	...	...	...	...	0.5	" " " " " "
" 18	390	19.6	7 3	2 70	"	...	...	...	14 2	...	" " " " " "
" 19	650	36 3	15 0	2.42	"	...	...	...	14 1	...	" " " " " "
" 20	400	21.0	10 3	2 04	Faint	...	...	...	...	0 5	" " " " " "
" 21	902	28.6	16 2	1.76	"	...	...	...	14 1	...	" " " 200 " "
" 22	919	27.8	16 6	1 54	Mod.	...	...	...	...	0.5	" " " " " "
" 23	1070	37.5	29 7	1 26	"	...	...	...	14 0	...	" " " " " "
" 24	689	23 8	10 2	2 57	Heavy	...	...	...	13 9	...	" " " " " "
" 25	885	27.6	15.7	1 76	Mod.	95	Faint	15 7	...	...	" " " " " "
" 26	642	29.3	7 0	...	V. Faint	...	...	...	13 8	...	" " " " " "
" 27	836	37.2	6.7	...	Faint	...	...	...	14 2	...	" " " " " " + 150 gm. starch.
" 28	752	26.2	9 0	...	"	...	...	...	14.5	0.5	500 gm. lung, 200 gm. suet.
Mar. 1	715	40.8	11.0	...	"	...	...	...	...	...	" " " " " "
" 2	770	30.9	10.8	2.87	V. Faint	...	...	...	...	...	" " " " " "
" 3	346	17.7	5 7	3.13	"	...	...	...	...	...	" " " " " "
" 4	551	17.6	...	...	...	...	...	...	13.7	...	" " " " " "
" 5	498	17.1	7.7	2.22	Heavy	87	Faint	13.9	13.3	...	" " " " " "

The experiment was planned for trial of a group of opposed factors. The diet was constantly in excess of the known tolerance, but phlorizin robbed the body of more sugar than would have been excreted on account of the diabetes alone. This sugar was entirely derived from protein, except toward the latter part of the experiment when starch was added to burden the tolerance still more severely. Under these circumstances, would the dietary excess, the formation of sugar from protein, etc., break down the tolerance or would the loss of sugar, due to phlorizin, protect the tolerance? Also, the diet was chosen from general experience as being something near a maintenance ration for a dog of this size, but the phlorizin caused a loss of weight comparable to what occurs in fasting. Under these circumstances it is improbable that any reduction of metabolism occurred, and it is even possible that the total energy exchange was somewhat increased. In other words, by this device the reduction of weight characteristic of fasting was obtained without the characteristic reduction of metabolism. Would there thus be a benefit to the tolerance like that produced by fasting, or not?

These questions could be definitely answered, because an overfeeding program of this duration (Dec. 18 to Jan. 11) in a severely diabetic dog possessing only one-twelfth or less of the pancreas will certainly cause a marked aggravation of the diabetes, so that ordinarily the glycosuria will no longer be controllable by fasting. The last injection of phlorizin was given on Jan. 10. The glycosuria persisted unusually long, and did not cease until Jan. 20, but the plasma sugar was constantly below 0.1 per cent., showing that the phlorizin was still responsible. Meanwhile, following Dec. 11, the protein ration was reduced successively to 300, 200 and 100 gm. of lung, while 200 gm. or more of suet was given daily in order to prevent further undernutrition as far as possible. Normal urine and blood sugar continued with increasing diet after Jan. 20. Jan. 25, the diet was 500 gm. lung and 200 gm. suet. The plasma sugar was 0.113 per cent., before feeding, and rose only to 0.172 per cent. after feeding, while glycosuria remained absent, demonstrating a higher tolerance than before the phlorizin period. Glycosuria continued absent on 800 gm. lung daily with variable quantities of suet, while the body weight rose from a minimum of 13.5 kg.

to 14.5 kg. On Feb. 5, the plasma sugar before feeding was 0.094 per cent., and 6 hours after feeding was 0.139 per cent., though this single determination may not have represented the maximum.

The entire experiment up to this point conforms to the rule that the tolerance of the diabetic organism is higher when the weight is reduced. This gain of tolerance was obtained on an actually excessive diet, merely by employing phlorizin to deprive the body of the surplus sugar. The benefit of fasting and reduction of weight was thus achieved without fasting and probably without the accompanying reduction of metabolism.

Probably a number of persons have had some idea of using phlorizin in the clinical treatment of diabetes. The experimental evidence indicates that its use might be feasible in a certain way, by enabling the patient to take a somewhat larger diet while preventing harm to the tolerance by means of this artificial withdrawal of sugar from metabolism. A practical application was never attempted, because of the greater danger of acidosis in human patients than in dogs, and because it appears unwise to create a pathological state, such as phlorizin poisoning, for no better purpose than a possible gratification of appetite.

Following Feb. 7, the experiment was continued for another purpose, namely, the comparison between the action of phlorizin in the later period, when diabetes was only latent, with its action in the earlier period when there had been active diabetes. Accordingly, the exact program of diet and phlorizin was duplicated, as shown in Table 2. The animal in the latter period behaved like a normal dog, showing lower D:N ratios and much lower quantities of excreted sugar and nitrogen than in the earlier period. This result is one of those discussed under section (1) above.

3. *Comparative Effects of Diabetes and Phlorizin Glycosuria.*

Acidosis was slight both in dog D4-60 and in dog D4-49, described below, and the differences were unimportant. A later publication (Series V, No. 5) will discuss the peculiarities of acidosis under such circumstances, and also add illustrations of the present topic.

Visible lipemia was absent or trivial in both of these animals. As previously mentioned,[16] however, the intense lipemia of some diabetic dogs and patients is never duplicated in phlorizin poisoning;

and though lipemia is not directly related to the insular function, the tendency to it constitutes another difference between diabetes and phlorizin glycosuria.

Totally depancreatized dogs are well known to lack the power of healing wounds or resisting infection to a remarkable degree which seems to be specific to the loss of pancreatic function, for it is not equalled in any other known condition. Partially depancreatized animals recover well from aseptic operations (except cats, which are strikingly deficient in wound healing when nine-tenths or more of the pancreas is removed). Dogs and other species, however, in the later cachectic stages of the diabetes following partial pancreatectomy often show a tendency to ulcers and other infections which apparently are analogous to clinical diabetic gangrene. None of these phenomena are explainable by loss of sugar. "Totally" phlorizinized dogs, though showing higher D:N ratios and higher absolute excretion of sugar than totally depancreatized dogs, withstand operations without anything resembling the prostration of healing and resisting power. Hyperglycemia, or the old idea of "sugar-soaked tissues," is likewise not an explanation, for normal animals subjected to prolonged excess of sugar show no such effects,[11] and the condition of depancreatized animals is not helped by phlorizin. Diabetic animals are peculiarly susceptible to phlorizin, in the sense that their blood sugar is reduced by surprisingly small doses. Illustrations are furnished in a previous paper[4] by the effects of doses as low as 0.2 or 0.3 gm., two or three days apart in the diabetic dog E5-99. Contrary to what might be expected, the plasma sugar was reduced lower in dog D4-60 (Table 1, above) by the same phlorizin dosage in the early period with active diabetes than in the later period with lower body weight and quiescent diabetes. It is similarly possible to control the hyperglycemia of totally depancreatized dogs by harmlessly small doses of phlorizin but there is not the slightest improvement in the healing or resisting power. This fact should arouse reflection in those who regard diabetes as nothing but a lack of glucose utilization.

Under such a doctrine, it also seems inexplicable that a totally depancreatized dog, if kept free from peritonitis, should still die within one or two weeks, when acidosis is absent or trivial and the animal should theoretically be able to live on non-carbohydrate materials until emaciated to a much greater degree than is found at autopsy. The asthenia of the diabetic dog also seems much more pronounced than that of a fasting phlorizinized dog under similar conditions. Investigators are mostly familiar with "totally" phlorizinized fasting animals, with the heavy drain upon their nutrition and with acidosis frequently severe. But if the doses of phlorizin are so chosen as merely to duplicate the glycosuria and acidosis of a depancreatized dog, the difference in strength is made more striking.

Depancreatized birds are known commonly to excrete little or no sugar, but will probably be found to die more quickly than phlorizinized birds which have greater glycosuria. Also occasional dogs from unknown causes excrete extraordinarily little sugar after total pancreatectomy, but do not live longer on this account. Further studies of the respiratory quotients in such dogs or in birds may be useful. The symptoms and death following pancreatectomy suggest the possibility that combustion of foods to the normal end products is not necessarily synonymous with normal or beneficial combustion. It is conceivable that the former may occur without insulin, while insulin may be indispensable for actually normal physiological combustion of protein, carbohydrate and fat. The direct participation of insulin in total metabolism is one possible hypothetical explanation why animals are so much worse off when deprived of insulin than when merely deprived of carbohydrate.

A further experimental opportunity seems to be offered by partially depancreatized dogs, which are able to digest and absorb food satisfactorily. A severely diabetic animal typically emaciates in spite of utmost eating and dies with profound weakness and cachexia. Suppose that a non-diabetic animal is placed on the same diet and given such phlorizin dosage as will duplicate the glycosuria, acidosis and any other known chemical abnormalities. Will there be a similar progressive impairment of health and ultimate death?

Several attempts were made to answer this question by parallel observations of diabetic dogs and normal controls treated with phlorizin, but many mishaps can arise to spoil experiments of such a character and duration. The most complete result was obtained

TABLE 3.

Dog D4-49.

Date	URINE					BLOOD PLASMA			Body Weight kg.	DIET
	Vol. c.c.	Sugar gm.	T. N. gm.	D N ratio	Acetone gm	Sugar mg per 100 c c	ACETONE			
							Qual.	Total mg. per 100 c c		
Dec. 21	860	Heavy	.. ...	.	..			. .	.. .	Bread and lung.
" 22	1051	66.2	.. ...	....	..		.	.	. .	800 gm. lung.
" 23	1492	91.0	.. ...	..	Mod.		.	.	.	" " "
" 24	1318	69.9	.. ..	....	.			.	.	" " "
" 25	755	33.2	. ....	.. .	Heavy	. .	..		.	" " "
" 26	1202	67.3	17.6	3.85	"	.	..			1 kg. lung.
" 27	1395	84.5	19.3	4.35	0.98				18.0	" " "
" 28	1210	37.9	16.1	2.35	0.85			.		" " "
" 29	880	46.4	20.2	2.30	0.84		.			" " "
" 30	961	34.6	14.8	2.35	0.96				.	500 gm. lung, 100 gm. suet.
" 31	466	21.9	7.5	2.87	0.68	.. ..	.	.	.	" " " " " "
Jan. 1	1045	40.8	16.1	2.54	1.48	384	Slight	27.0	. .	" " " " " "
" 2	1470	38.2	13.2	2.89	1.59	.	.		15.9	" " " " " "
" 3	688.	37.2	11.2	3.32	1.38	435	Slight	41.5	. . .	" " " " " "
" 4	318	22.9	8.8	2.60	0.93	.. ..	.. .	..	15.1	" " " 200 " "
" 5	1315	38.1	18.5	2.06	1.37	......	.... .	. . .	...	" " " " " "
" 6	982	20.6	12.4	1.66	0.77	.	.....	.. . .	. . .	" " " " " "
" 7	1120	31.4	8.3	3.79	0.93	435	Heavy	52.9	.....	" " " " " "
" 8	784	21.0	5.5	3.82	0.67	. ...	.... .	......	.. ...	" " " 100 " " and 50 gm. corn starch.
" 9*	1140	52.4	6.5	......	0.73	455	......	21.7	13.6	500 gm. lung, 200 gm. suet.
" 10	490	23.3	8.3	......	0.78	......	......	. ..	......	" " " " " "
" 11	225	12.8	3.8	......	0.29	75	......	22.0	13.0	" " " " " "

* 0.3 gm. phlorizin injected subcutaneously.

in an experiment with dog D4-49, for which dog D4-60 served as a control. Here the most extreme possible conditions were chosen, in two respects. First, both dogs were partially depancreatized, so as to equalize digestive conditions as far as possible; and as the diabetes in dog D4-49 had been brought on partly by inflammation, the pancreas remnant was larger than in dog D4-60 and it was hoped the digestion would be correspondingly better. Second, the control dog, D4-60, was actually diabetic, and, as explained under section (2) above, the diabetes had merely been controlled and converted into a different form of glycosuria by means of phlorizin. Could this difference be further demonstrated by the different influence upon the animal, even when the quantities of sugar and nitrogen lost were similar?

The operative record of dog D4-49 was previously published.[12] He was somewhat stronger and heavier than dog D4-60, and weighed 24 kg. on Nov. 24, 1916. On this date 28.5 gm. of pancreatic tissue was removed, leaving the body of the gland, estimated at 15 gm. Glycosuria remained absent until the pancreas remnant was traumatized by crushing in a second operation on Dec. 7. The dog was then unwell and free from glycosuria on account of nearly complete fasting until Dec. 12. With return of appetite and spirits, the eating of considerable bread on that day brought on heavy glycosuria. This continued on a diet of bread and meat, until on Dec. 22 the dog was placed on carbohydrate-free diet for exact comparison with dog D4-60. Owing to the anorexia, this dog had lost more weight than dog D4-60 up to this point, but nevertheless at the beginning of the parallel test dog D4-49 showed distinctly the better strength and general condition of the two.

The pancreatic islands of dog D4-49 were evidently profoundly injured by the inflammation, for they quickly succumbed to hydropic degeneration and at autopsy only rare disappearing remains were found. Correspondingly, the diabetes was not far from "total," as indicated by the D:N ratios (Table 3). The operative wound healed smoothly, but the decline of weight and strength was almost as rapid as after total pancreatectomy. The prostration rapidly became extreme, and unusually early death occurred in profound asthenia on Jan. 11, while dog D4-60 was still in fair strength and spirits. Two days before death (Jan. 9), dog D4-49 was given an injection of 0.3 gm. of phlorizin, which reduced the blood sugar but did not alter the moribund state.

Comparison of Tables 1 and 3 shows that the urinary sugar and nitrogen and D:N ratios of dog D4-49 were fairly comparable with those of the first period of dog D4-60, and the differences in the clinical course are not explainable by any excess of excretion on the part of the former dog. Also, the degree of acidosis was unimportant in both animals. The plasma bicarbonate, which had been normal, fell to 36.6 volumes per cent. in dog D4-49 before death, but there were no symptoms resembling coma, the total acetone of the plasma was too low to be compatible with such a diagnosis, and the reduction of blood alkali was therefore evidently nothing but a perfectly familiar antemortem occurrence.

Totally depancreatized dogs seem to die before reaching a degree of emaciation equal to that of normal animals which starve to death. But in dog D4-49 and other partially depancreatized dogs, the loss of weight may be sufficient to account for death. Dog D4-60 weighed only 13.3 kg. at the close of the second phlorizin period and was still far from a dangerous condition. Also, the question is still open why a diabetic dog should be weaker than a phlorizinized dog when the weights are similar. Nevertheless, it must be recognized that dog D4-49 lost more weight both absolutely and relatively than the control, and the fatal weakness is at least very largely thus explained.

The diets of the two animals were identical. The feces of both were saved, but circumstances prevented the analyses for nitrogen and fat which had been planned. From the gross appearances it could be inferred that the digestive power of both was closely similar at the outset, but toward the close there may have been progressive failure of digestion in dog D4-49, and this may account for the falling of his urinary nitrogen. This point, however, is not essential. Only respiration tests could prove whether the more rapid wasting of the diabetic dog is due in any degree to a higher metabolism. It would not be surprising if the general failure of bodily powers in a diabetic animal should include a failure of digestion and absorption, and this difference from a phlorizinized animal will be significant if it is constant. The general experience with other observations of this kind, which were not sufficiently complete to report in detail, gives the impression that the difference is constant. Dogs receiving adequate rations of carbohydrate or protein can apparently endure phlorizin glycosuria almost indefinitely without serious harm, while diabetic dogs with similar nitrogen and sugar excretion weaken and die. These observations agree with other evidence of a fundamental difference between the two conditions.

SUMMARY AND CONCLUSIONS.

1. Partially depancreatized dogs whose diabetes is controlled by diet react to phlorizin like normal animals. Dogs with active diabetes respond to phlorizin with exaggerated sugar and nitrogen excretion. This behavior is in harmony with the idea of an abnormal tendency to tissue breakdown when insulin is lacking, though it does not prove this hypothesis.

2. When, under suitable conditions, diabetic animals are given a diet in excess of the tolerance, but at the same time are robbed of the excess sugar by means of phlorizin, no impairment of the tolerance results. The loss of weight caused by phlorizin actually raises the tolerance, like ordinary undernutrition. Three deductions are warranted by these experiments:

(a) Phlorizin does not injure the islands of Langerhans.

(b) The digestion and absorption of either carbohydrate or protein, the formation of glucose from protein, the transportation of sugar in the blood and its excretion by the kidneys create no demonstrable demand upon the insular function. This demand seems to arise only from the combustion or storage of sugar or other food.

(c) The rise of tolerance which characteristically accompanies a fall of body weight seems to be obtainable without a reduction of metabolism, since the metabolism during phlorizination probably does not fall and perhaps rises. The benefit of undernutrition would therefore seem to consist chiefly in the reduction of body mass. This agrees with other evidence that the maintenance of the entire body mass somehow constitutes a load upon the insular function.

3. Totally depancreatized dogs show loss of healing and resisting power, asthenia, and death before a state of utmost emaciation is reached. Fasting dogs receiving sufficient phlorizin to produce an equal excretion of sugar and nitrogen remain in far better condition. Likewise, partially depancreatized dogs weaken and die with active diabetes, while phlorizinized controls receiving the same diet and excreting similar quantities of sugar and nitrogen survive. Hyperglycemia does not explain the differences, for phlorizin fails to save the diabetic dogs, and in this connection it is noted that surprisingly small quantities of phlorizin suffice to cause hypoglycemia in diabetic animals. Three further conclusions are suggested:

(a) Phlorizin poisoning is a radically different condition from diabetes. The word diabetes is long and thoroughly established as the name of the condition which results from deficiency of insulin. It therefore appears confusing and scientifically unjustifiable to apply this name to a wholly different condition.

(b) Diabetic symptoms and death are not explainable wholly by the loss of sugar. Animals are much worse off when deprived of insulin than when merely deprived of carbohydrate.

(c) All the evidence seems to characterize diabetes not as a mere deficiency of sugar metabolism but as a specific defect of the total bodily nutrition.

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THE QUESTION OF SENSITIZATION OF JOINTS WITH NON-HEMOLYTIC STREPTOCOCCI.

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These experiments were planned in an attempt to substantiate the hypothesis that the arthritis of acute rheumatic fever is the result of a preliminary specific sensitization of the joints by non-hemolytic streptococci. This hypothesis has been advanced by both Herry (1) and Faber (2), and accepted by certain authors as the most probable explanation of the peculiar type of inflammation found in the joints of patients with this disease.

Herry claims that the peculiarity of the rheumatic lesions is due to an "*endotoxine rhumatismale*" which, he states, he was able to prepare from streptococci grown on solid media by a process of drying, grinding, and extracting with normal saline, and finally removing the bacterial bodies by centrifugalization. He states that the extract from 2 mg. of streptococci was fatally toxic for rabbits, and that the introduction of a few drops of clear extract into the joints of rabbits sensitized these structures so that intravenous inoculation of the animals with homologous streptococci after 8 to 15 days was constantly followed by arthritis in the sensitized joints.

In view of the difficulty experienced by most workers in obtaining endotoxins from any type of streptococci, the claim of Herry that he could produce a powerful toxic substance from a small amount of non-hemolytic streptococcus culture is most surprising. Faber, indeed, was unable to repeat successfully his experiments; and we have likewise failed to obtain an endotoxin from these cocci. Faber, on the other hand, reported that by injecting rabbits' knees with killed cultures of non-hemolytic streptococci these joints were rendered more liable to involvement following subsequent intravenous inoculation with homologous living microorganisms. He, unfortunately, does not state whether the "sensitized joints" were the only joints involved. Because preliminary injection of rabbits' knees with killed pneumococci or typhoid bacilli did not render these structures sensitive to a subsequent intravenous inoculation of the animals with non-hemolytic streptococci, he claimed that "this preparatory or sensitizing process is, within narrow limits, a strictly specific one." It should be pointed out that in his experiments arthritis did not occur in animals first injected into the joints with killed pneumococci or typhoid bacilli and subsequently inoculated intravenously with homologous living bacteria. If the pneumococcus-treated animals are eliminated from his protocols, cross-sensitization was obtained in only two out of four rabbits instead of two out of ten.[1] This makes it appear that the so called *sensitization* in his experiments was the production of a local lesion which upon healing left a *locus minoris resistentiæ*, a place favorable for the localization of bacteria having a tendency to produce joint lesions. When pneumococci or typhoid bacilli, having little tendency to produce arthritis, were inoculated intravenously into rabbits with joints previously injected this *locus minoris resistentiæ* was not made evident. This seems to us the most probable explanation of Faber's experiments. It is not sufficient, however, to explain the fact that his rabbits inoculated intravenously three times with "Streptococcus 7" had arthritis more frequently than those inoculated only once or twice. We think it most probable that small areas of inflammation were set up in the joints of rabbits following the first or second inoculation, and that these small lesions produced points of lessened resistance. Then, if another strain of streptococci had been used for the third inoculation, joint lesions would have been expected with the same frequency as if the homologous strain had been introduced.

Another way to determine this point would be to inject the right knees of several rabbits with one strain of streptococci and the left with another, and subsequently to inoculate half of the animals with one of the strains and the remainder with the other. If arthritis occurred only in the knees of animals in which the same strain was used for both intraarticular injection and intravenous inoculation one might conclude that specific joint sensitization had been induced. If, on the other hand, arthritis occurred in both knees or in other joints, the probability that specific sensitization had taken place would be seriously doubted. In this manner we attempted to elucidate the question.

EXPERIMENTAL.

Method.

In all instances the experimental animals were brown rabbits weighing 1,200 to 2,000 gm. The hair over the knees was clipped closely so that the bony prominences could be easily recognized and the joints minutely inspected. In order to have a more accurate record of the degree of swelling of the knees these joints were measured daily as follows: the widest distance between the internal and external condyles of the femora, and the broadest portion of the upper end of the tibiæ. As these measurements were over bony prominences it was fairly easy to have them comparable from day to day. They were made with special calipers having a vernier scale reading to 0.1 mm. It may be thought that such measurements are too fine for practical purposes and that conclusions based on them are liable to be faulty, but considerable preliminary work satisfied us that repeated measurements on normal animals are constant to within 0.2 to 0.5 mm., and frequently there is practically no variation; hence it seemed justifiable to record the degree of joint injury in this manner. Notes were also made of redness, swelling, heat, and stiffness. The last symptom, when occurring early, was regarded as a measure of pain; when occurring later, as an indication of the amount of permanent injury. For the first 5 or 6 days following the intraarticular injections the animals were inspected daily, then every 2nd day for about 10 days, and subsequently twice a week until they were inoculated intravenously; then daily inspections were resumed.

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[1] Faber (2), Table VI.

In the first three experiments, after preliminary measurements of the knees, these joints were injected with killed streptococci in the amounts indicated in the protocols; the suspensions were prepared as follows: The centrifugates of 18 to 24 hour broth cultures of the cocci were suspended in normal saline and heated ½ hour at 56°C., then tested by plating to insure sterility. The suspensions were so diluted that 0 5 cc. contained the desired amount of streptococci. Most of the joints were injected with an amount corresponding to 0.5 cc. of the original broth culture, as it had been determined in previous experiments that this amount of heat-killed, non-hemolytic streptococci could be injected into the knee joints of rabbits without demonstrable permanent damage. The injections were made according to the method described by Faber; the needle was passed in a proximal direction through the middle of the patellar tendon until it was felt to slip into the bursa under the patella; the suspension was then slowly introduced, a small amount withdrawn into the syringe by suction and reinjected (piston test) in order to be sure that the needle was properly placed; the needle was then quickly withdrawn to prevent leakage into the periarticular tissue. When it was certain that the bacteria had been injected into this tissue the animals were discarded. The bacteria for intravenous inoculation were usually grown for 18 hours in 10 per cent rabbit serum broth, centrifugalized, and resuspended in sterile Ringer's solution. Except with the first culture of Strain Z75, the streptococci had been recently passed several times through rabbits' knees in an attempt to increase their virulence.

The streptococci were all isolated from blood cultures of patients with rheumatic fever, were typical *viridans* strains possessing different biochemical and immunological characteristics, and had decreasing degrees of virulence as follows: A49, A135, 38D, and Z75.

At the postmortem examination the degree of joint involvement was noted, and films from all the joints were prepared and examined microscopically. In some instances there seemed to be an increased amount of clear fluid, but films revealed no exudation of cells; hence these joints were considered normal. Four degrees of inflammation were distinguishable.

Degree.	Gross appearance.	Microscopic appearance.
+++	Yellow, turbid, purulent fluid.	Marked increase of exudative cells.
++	Gray, cloudy or hazy fluid.	Distinct to marked increase of exudative cells.
+	Increased clear fluid.	Distinct increase of exudative cells.
±	Normal amount, clear fluid.	" " " " "

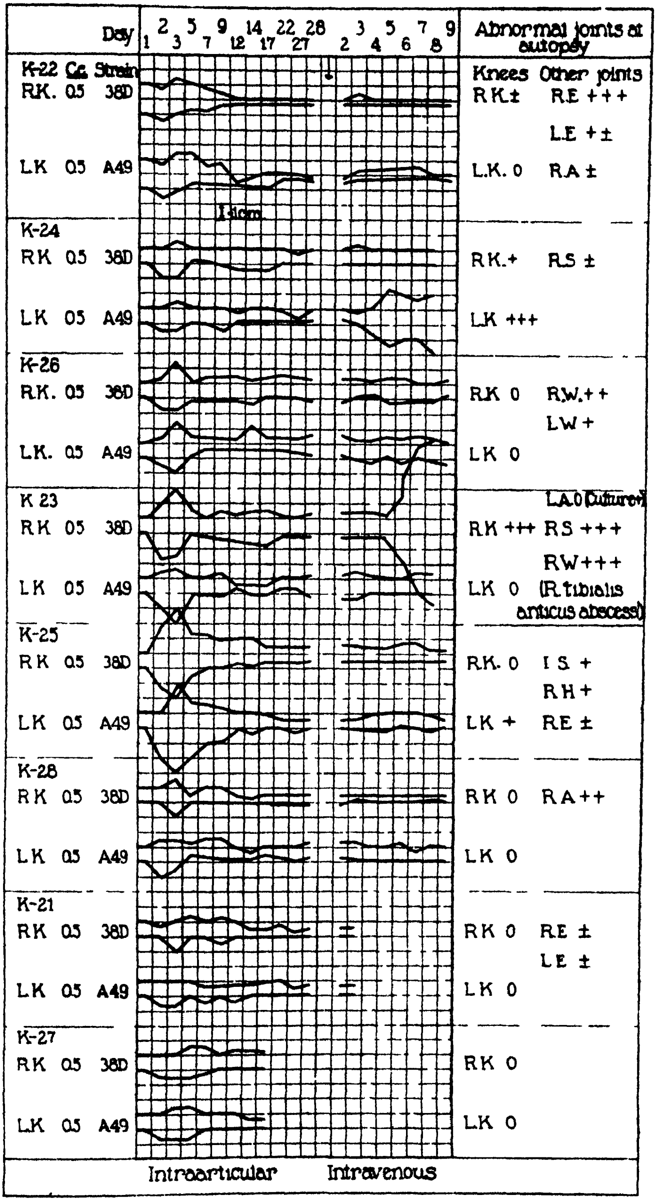
Except in Experiment 4, half of the rabbits received sodium salicylate by stomach tube from the day previous to intravenous inoculation until the time of death. This was done to study the effect of salicylates upon possible joint sensitization, for if bacterial sensitization is the important factor in determining the localization of rheumatic arthritis it would seem probable that salicylates would exert a profound antiarthritic influence. In another communication (3) we have shown that sodium salicylate apparently decreases the intensity of the arthritis in some rabbits inoculated intravenously with non-hemolytic streptococci.

If one considers only the knee involvement among the animals in Experiments 1 and 2, here reported, it would seem as though the salicylates might have exerted an inhibitory influence upon joint sensitization; but consideration of all the inflamed joints makes this less probable.

Explanation of Text-Figures.—The upper line for each knee shows the change in the measurement of that joint through the condyles of the femur; an upward direction of the line indicates increase, a downward direction, decrease. The lower line for each knee shows a change in measurement through the broadest portion of the upper end of the tibia; but here a downward direction of the line indicates an increase, and an upward direction, decrease. The area between these two lines indicates, therefore, the variation in the size of the knees. Broken lines indicate that no measurements were made on that day.

In each chart the blank space between the two periods of observation indicates that this was the last day of the "intraarticular period" and the first of the "intravenous."

R. K. indicates right knee; L. K., left knee; other joints are designated in a similar manner by the initial of the side and joint.



Text-Fig. 1. Measurements of knees following intraarticular injection and intravenous inoculation. The arrow indicates the day on which the animals received an intravenous inoculation of the sediment of 9.3 cc. of Streptococcus 38D/4/2.

Experiment 1.—The knee joints of eight rabbits were injected with the sediment of 0.5 cc. of broth culture of killed streptococci as follows: right, with Strain 38D/3/4;[2] left with Strain A49/8/4. The animals were observed for 4 weeks, when it was evident that the inflammation resulting from the intraarticular injection had entirely subsided. 17 days after this first injection, Rabbit K-27 died, evidently from an intestinal infection; both knees appeared normal, and the synovial fluids were free from cellular exudate. This confirms the conclusion derived from clinical observation of the knees, that the injury resulting from the introduction of killed streptococci into them did not persist more than 3 weeks. Salicylate treatment was started with Rabbits K-21, K-23, K-25, and K-28 on the 27th day, and on the next day each of the seven surviving animals was inoculated intravenously with the sediment of 9.3 cc. of serum broth Culture 38D/4/2. The animals were then inspected daily until the 7th, 8th, or 9th day when they were sacrificed and autopsied. Films and cultures were made of all the joint fluids (Text-fig. 1).

Text-fig. 1 shows graphically the reaction of the knees following both the intraarticular injections and the intravenous inoculations and also the degree of inflammation in all abnormal joints at the time of autopsy. Although the animals responded differently to the intraarticular injection, each animal showed a similar amount of swelling in the two knees. It seems evident, therefore, that the two strains of streptococci, when killed, were equally irritating. This is noteworthy when compared with the results obtained from inoculating the two strains intravenously; Strain A49 is much more virulent than Strain 38D. They have different fermentation reactions and are distinct immunologically. One would think, therefore, that if joints were specifically sensitized by having previously reacted to one strain, the intravenous inoculation with that strain would always result in a recurrence of arthritis in that joint, and the opposite knee having previously reacted to the other strain would remain free from a second reaction. This expected result, however, was not obtained in this experiment. Rabbit K-21, dying 2 days after the intravenous inoculation from a Gram-negative bacillus infection, showed no evidence of involvement of either knee, but had slight arthritis in both elbows. Among the six animals surviving the full period of the experiment there were three inflamed right knees,—the joints injected with the same type of bacteria subsequently used for intravenous inoculation; there were also two inflamed left knees,—the ones previously injected with the other strain; and, in addition, there were twelve other inflamed joints among these animals; also one joint, showing no cellular reaction, from which a pure culture of Streptococcus 38D was grown; this must be considered evidence of bacterial invasion of the ankle in this rabbit (No. K-23). In the seven animals, twenty joints were, therefore, involved, but only three of these were previously injured with the same type of streptococcus that was used for intravenous inoculation. Four knees so injured failed to show any abnormalities post mortem. The knees of one animal (No. K-25) appeared slightly stiff for 3 days after the intravenous injection; this stiffness was thought to be a residuum from the inflammation following the initial joint injury. Both knees of this rabbit showed a marked reaction to the intraarticular injection, but the left, first injured by Streptococcus A49, was the one invaded following the intravenous inoculation with Streptococcus 38D, and three other joints of this animal were also acutely inflamed. Rabbits K-22 and K-23 might be cited as examples of specific joint sensitization if only the knees were considered, but the involvement of three other joints in each animal renders this position untenable.

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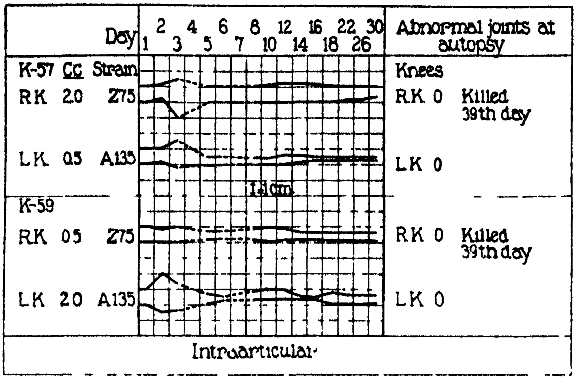
[2] The first figure indicates the strain of streptococcus; the second, the number of passages through rabbits' joints; and the third, the number of subcultures since the last animal passage.

There seemed to be slight, if any, relationship between the amount of gross reaction following the initial joint injury and the subsequent localization of the living bacteria. By this we do not mean to imply that severe injury to a joint would not be a factor in determining localization of microorganisms in that joint, but rather that the amount of local injury resulting from the intraarticular injection in the rabbits was too slight to have unfavorably influenced the reacting power of the knees. It is probable that only by producing such slight injuries is it possible to study the question of bacterial joint sensitization, for if more severe trauma were inflicted a *locus minoris resistentiæ* would result and doubtless favor the localization of many types of microorganisms circulating in the blood stream.

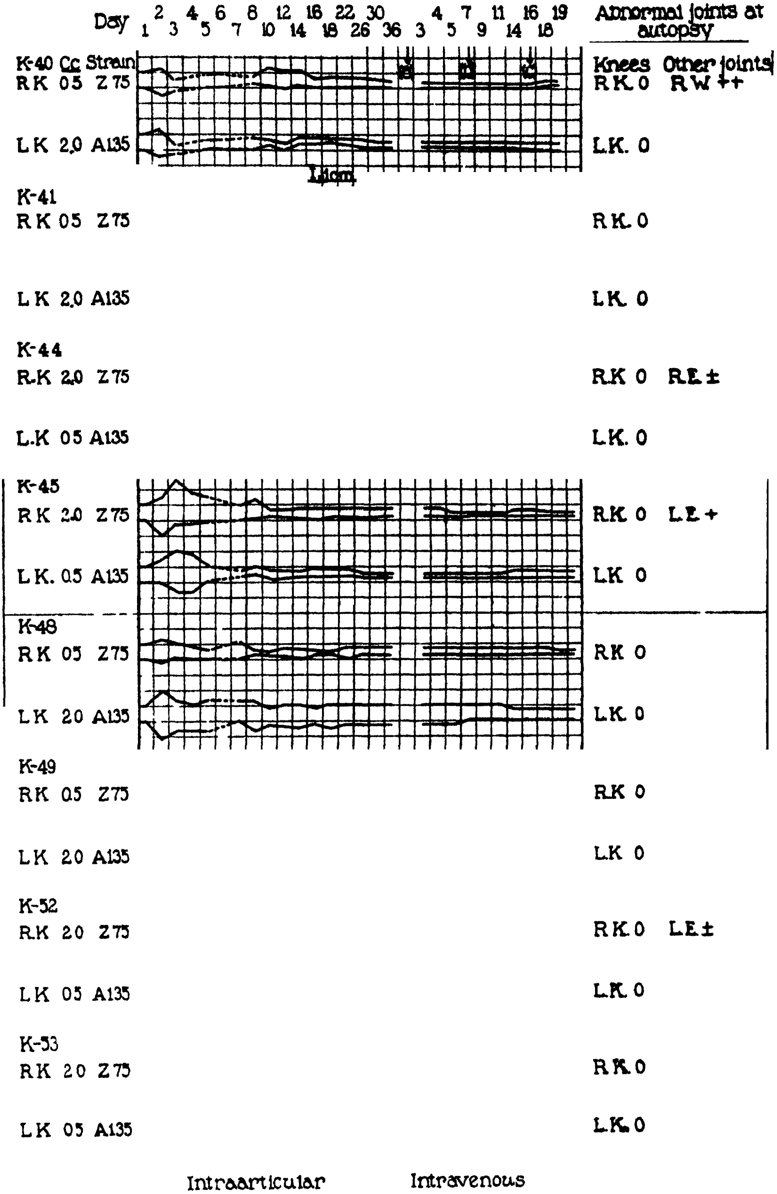
The cultures from the synovial fluids of all the joints were sterile, with the exception of those from the right knee and the left ankle of Rabbit K-23. The upper half of the right tibialis anticus, and extensor longus digitorum muscles of this animal were markedly inflamed and yielded a small amount of purulent material on incision. These findings indicate that the animal had comparatively little natural resistance to the infection.

Experiments 2 and 3 serve as controls for one another in that the animals received intraarticular injections of the same streptococci: Strain Z75 in the right knees, and Strain A135 in the left; in half the animals the sediment of 0.5 cc. of broth culture of Streptococcus Z75 and 2 cc. of Streptococcus A135 were used; in the other half the amounts of these two streptococci were reversed. Two animals (Nos. K-57 and K-59) were controls in both experiments; their knee joints were injected in the same manner as in the others and observed for 39 days; then the rabbits were sacrificed and the joints found to be normal (Text-fig. 2). In these two rabbits the knee receiving the larger amount of culture showed the greater reaction, which would be expected; but both knees of many of the animals in the two experiments showed reactions of similar intensity.



TEXT-FIG. 2. Measurements of knees of control animals following intraarticular injection with Streptococcus Z75 and Streptococcus A135.

Experiment 2.—The knees of eight rabbits were injected, and observed for 36 days, when it was certain that they had recovered from the local injury. On the 35th day half the animals (Nos. K-48, K-49, K-52, and K-53) were given 0.2 gm. of sodium salicylate per kilo of body weight; this treatment was continued until the termination of the experiment. On the 36th day all the rabbits were inoculated intravenously with the sediment of 50 cc. of broth Culture Z75/0/11; 7 days later none showed any evidence of joint involvement, so they were reinoculated with the same amount of Culture Z75/1/2; 8 days later they still failed



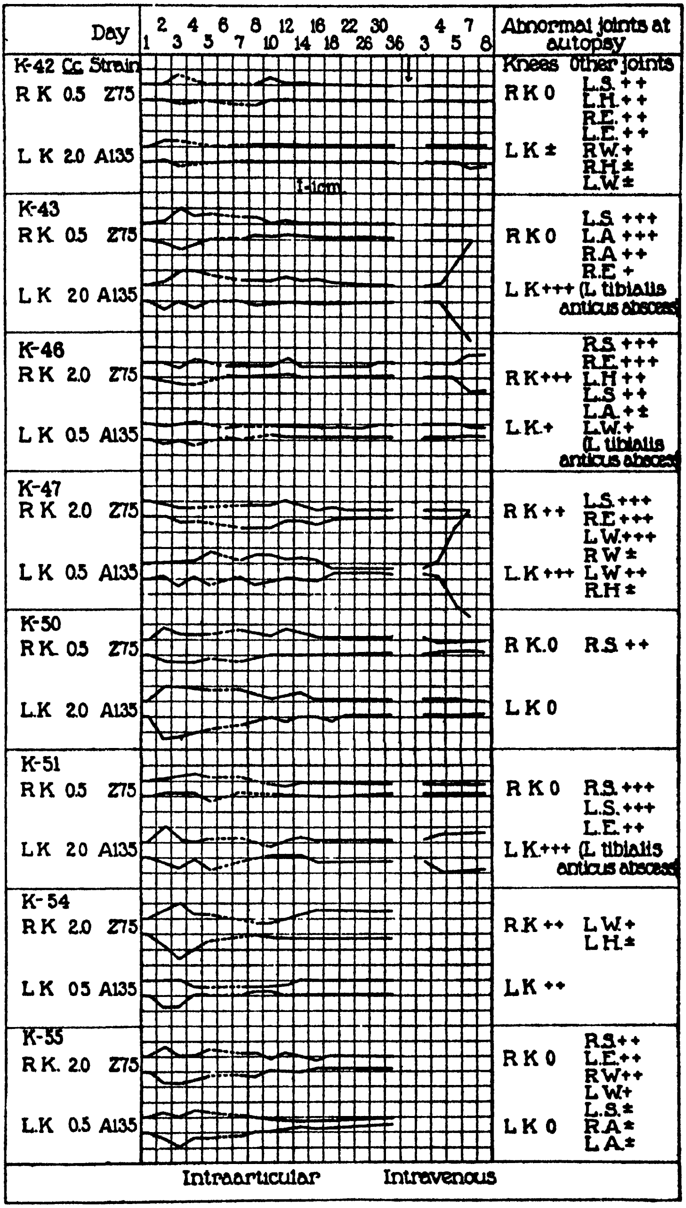
Text-Fig. 3. Measurements of knees following intraarticular injection and intravenous inoculation. The arrows indicate the days on which the animals received intravenous inoculations of (*a*) the sediment of 50 cc. of Streptococcus Z75/0/11, (*b*) the sediment of 50 cc. of Streptococcus Z75/1/2, and (*c*) the sediment of 50 cc. of Streptococcus Z75/3/2.

to show any effect from the two inoculations, so were similarly inoculated with 50 cc. of Culture Z75/3/2. 3 days later all appeared well and clinically free from joint lesions; they were sacrificed and examined in the usual manner. The results are shown in Text-fig. 3.

Although most of the injected knees showed some degree of reaction following local injury, none gave any evidence of arthritis following three intravenous inoculations of the animals with large amounts of Streptococcus Z75. Among the eight rabbits, only four joints were found to be definitely diseased. This proved, however, that the inoculum, in the large amounts employed, was sufficient to incite arthritis. The primary injury produced in the right knees of all the animals by injection of this coccus would seem to furnish perfect conditions for the demonstration of specific joint sensitization. The observation that rabbits have more arthritis following repeated intravenous inoculations with non-hemolytic streptococci of low virulence is claimed by Faber to furnish further proof of joint sensitization. Both of the above mentioned conditions, *viz.* intraarticular injection of cocci and repeated intravenous inoculation, were fulfilled in this experiment without any evidence of such sensitization. It will be noted that the salicylated animals had fewer abnormal joints than the non-salicylated; this may be advanced as an argument in favor of eliminating these four animals from consideration, but lack of inflammation in any of the knees of the other four animals, and the results with salicylated animals in other experiments seem of sufficient weight to warrant the inclusion of all in this report.

Experiment 3.—Eight animals were used in this experiment; the knees were injected in the same manner and with the same cultures as those of Experiment 2. On the 35th day half of them (Nos. K-50, K-51, K-54, and K-55) were started on sodium salicylate. On the 36th day all were inoculated intravenously with the sediment of 30 cc. of serum broth Culture A135/2/2. The following day two of the salicylated rabbits (Nos. K-54 and K-55) died; the rest survived for 7 or 8 days, when they were sacrificed and examined as usual. The results are shown in Text-fig. 4.

Streptococcus A135 was much more virulent than Streptococcus Z75 and, therefore, furnished another type of experimental condition. In the entire group there were forty-five abnormal joints, an average of 5.6. The two rabbits (Nos. K-54 and K-55) dying the day after



TEXT-FIG. 4. Measurements of knees following intraarticular injection and intravenous inoculation. The arrow indicates the day on which the animals received an intravenous inoculation of the sediment of 30 cc. of Streptococcus A135/2/2. Rabbits K-54 and K-55 died the following day.

the intravenous inoculation had eleven inflamed joints, an average practically the same as that which prevailed in the surviving group, so it seems justifiable to include them in the general discussion. All the knees injured by intraarticular injection seemed to have recovered completely by the end of the 3rd week; at least, there was no appreciable change in size after that time. There was no constant relation between the reaction of the knees to intraarticular injection and the liability for any given joint to be involved after the intravenous inoculation; for example, both knees of Rabbit K-50 showed the most severe and persistent reaction, and neither of them was diseased at the time of death. From this animal alone one might conclude that the preliminary articular injury had led to a condition of immunity, but the results in Rabbit K-43 point in the other direction. Both knees of this animal showed intense and fairly persistent swelling following the initial injury, but only the left was involved following the intravenous inoculation; four other joints of this animal were also inflamed, and there was an abscess in the left tibialis anticus muscle. So many foci

TABLE I.

Summary of Joints Involved in Experiment 3.

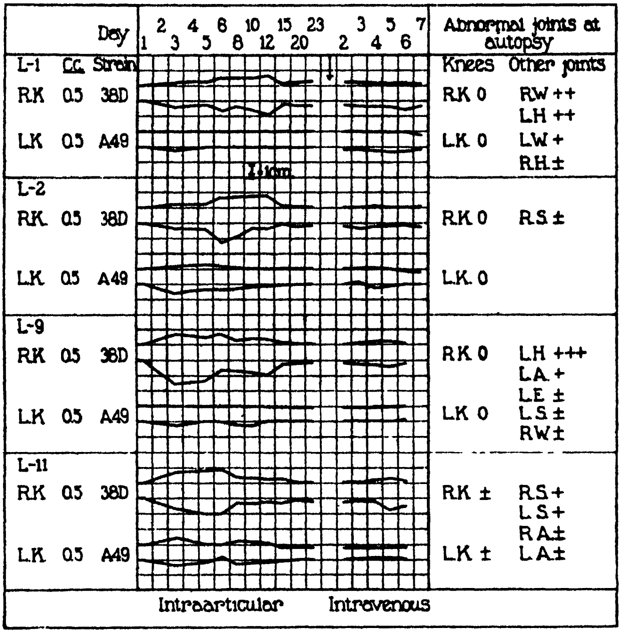
Joint.	Rabbit No.								Total involved	Per cent involved.
	K-42	K-43	K-46	K-47	K-50	K-51	K-54	K-55		
R. S.	0	0	+	0	+	+	0	+	4	50
L. S.	+	+	+	+	0	+	0	+	6	75
R. E.	+	+	+	+	0	0	0	0	4	50
L. E.	+	0	0	+	0	+	0	+	4	50
R. W.	+	0	0	+	0	0	0	+	3	37
L. W.	+	0	+	+	0	0	+	+	5	62
R. H.	+	0	0	+	0	0	0	0	2	25
L. H.	+	0	+	0	0	0	+	0	3	37
R. A.	0	+	0	0	0	0	0	+	2	25
L. A.	0	+	+	0	0	0	0	+	3	37
R. K.	0	0	+	+	0	0	+	0	3	37
L. K.	+	+	+	+	0	+	+	0	6	75

of infection indicate that the animal had much less resistance than did No. K-50.

A summary of the distribution of arthritis among the joints of the different rabbits (Table I) shows that the left knee—the one previously injected with Strain A135—was involved in six animals; the right—previously injected with Strain Z75—was invaded half as frequently. If we consider the percentage of arthritis in the various sets of joints, *i.e.* right shoulder, left shoulder, etc., it is evident that the left shoulder was involved as frequently as the left *sensitized* (?) knee, and that in most instances more joints were affected on one side of the body than on the other. Two of the animals failed, in addition, to show arthritis in either of the previously injected joints; it would, therefore, be illogical to conclude that the localization of inflammation in the left knees of the other six was due to a specific streptococcus joint sensitization. We are inclined to regard the localization in the knees as due to the same factors that determined the localization in other joints—virulence and invasive properties of the streptococci.

Because of the apparent failure to induce joint sensitization with heat-killed streptococci an attempt was made in one experiment to sensitize joints with an extract of streptococci prepared by the method used by Herry. This was considered advisable because of the theory of Zinsser (4, 5) that substances of much smaller molecular size than bacteria may penetrate cells and lead to an allergic state of these cells. It is evident that the experiment as planned by us is not of the same nature as those usually performed to demonstrate sensitization; in the latter the animal is sensitized by introduction of the antigen intravenously or intraperitoneally, possibly subcutaneously, and the local allergic state demonstrated subsequently by introducing the antigen into some particular tissue, as the skin or mucous membrane. This local hypersensitiveness is only a part of a general allergy and cannot be considered the same as the "joint sensitization" described by Herry and Faber.

Experiment 4.—An extract of Streptococcus 38D and one of Streptococcus A49 were prepared as follows: Cultures were seeded on glycerol potato agar to which rabbit blood had been added. After 18 hours the growth was carefully scraped off, taken up in a small amount of saline, and desiccated *in vacuo* over P_2O_5 or KOH. Each extract consisted of two lots; one required 48 hours for desiccation and the other only 2 hours. The dried bacteria were then pulverized by grinding in an agate mill for 24 hours. The powder was weighed and mixed with sodium chloride solution and water in such proportions that each cubic centimeter contained 4 mg. of dried bacteria and 8 mg. of NaCl. The bacterial suspension was allowed to stand at room temperature with frequent shaking of the container for another 24 hours, when it was centrifugalized at high speed until water-clear. The supernatant solution was pipetted off; 0.5 cc. of Strain 38D was injected into the right knee of each of four rabbits, and 0.5 cc. of Strain A49 into the left. Cultures of the extracts showed that Strain 38D was quite heavily contaminated with white non-hemolytic staphylococci, and Strain A49 contained a very few of the same contaminants. No streptococci were recovered from either solution. The local reaction induced by the intraarticular injection might, therefore, have been due to the presence of contaminating staphylococci, as well as to the streptococcus extract. Indeed, the more marked swelling of the right knees when compared with the left suggests that the heavier contamination of Solution 38D was responsible for the severe reaction. It was shown, however, by marked turbidity of the solutions on boiling that they contained a large amount of bacterial protein.



TEXT-FIG. 5. Measurements of knees following intraarticular injection and intravenous inoculation. The arrow indicates the day on which the animals received an intravenous inoculation of the sediment of 5 cc. of Streptococcus A49/0/10.

Because of the contamination with staphylococci the solutions were filtered through a Berkefeld filter, and subsequently shown to be sterile. Their toxicity was tested as follows: one rabbit was injected intravenously with 3 cc. of Strain 38D, another with 3 cc. of Strain A49, and a third with 2 cc. of each. None of these animals showed any toxic symptoms. This result would be expected from our general knowledge of the lack of endotoxin in members of the coccus group of bacteria.

23 days after the four rabbits had received intraarticular injections of the two bacterial extracts, each was inoculated intravenously with the sediment of 5 cc. of culture of Streptococcus A49/0/10. They were observed for a week, then sacrificed and examined in the usual way. The results of this experiment are shown in Text-fig. 5.

As already noted, the right knees of all the rabbits reacted quite violently to the intraarticular inoculation. This was probably due to the contamination of the extract. The left knees injected with Streptococcus A49 extract, on the other hand, reacted only mildly. Therefore, at the time of intravenous inoculation the right knee of each animal had been intensely inflamed and possibly sensitized to Streptococcus 38D, and the left knee possibly sensitized to Streptococcus A49. The intravenous inoculation with Streptococcus A49 would be expected to bring out evidence of this sensitization if it existed. The dose was fortunately proper to induce arthritis in all the animals, but only one of them (No. L-11) had inflammation of the knees. Among the four animals there were sixteen inflamed joints, and among these the wrists shoulders, hips, and ankles were affected more frequently than the knees. It is evident, therefore, that the preliminary irritation of the knees of these rabbits did not render them especially liable to localization of the streptococci. The results in general agreed with those of the previous experiments.

DISCUSSION.

A summary of all the results is given in Table II, in which the order of the experiments is arranged according to the percentage of total joints involved in each group. This shows that there was provided a fairly wide range of conditions in respect to virulence and power of the various streptococci to invade joints. In two experiments (Nos. 2 and 4) the injected knees showed less tendency to be involved than did other joints, and the knee previously injected with killed streptococci or bacterial extract of the same strain that was inoculated intravenously reacted in exactly the same manner as the opposite knee that had been previously injected with another strain of streptococci. In another experiment (No. 1) the knees injected with homologous cocci were involved slightly more often than those injected with heterologous cocci, but the fact that the larger proportion of injected knees failed to be involved following intravenous inoculation of the animals shows that inflammation of some of the knees cannot be attributed to sensitization. In the remaining experiment (No. 3) twice as many knees injected with the homologous strain were involved compared with those injected with the heterologous strain; but Streptococcus A135 in the amount inoculated had such high invasive powers that practically half the joints of the rabbits were inflamed. It therefore hardly seems justifiable to attribute the involvement of the injected knees to a specific sensitization.

TABLE II.

Summary of the Results of All Experiments.

Experiment No.	2	1	4	3	Total.
No. of animals	8	7	4	8	27
Total No. of joints (12 per animal)	96	84	48	96	324
Total No. of joints involved	4 = 4%	20 = 24%	16 = 33%	45 = 47%	85 = 26%
Total homologous "sensitized" knees involved (1 per animal)	0	3 = 43%	1 = 25%	6 = 75%	10 = 37%
Total non-homologous "sensitized" knees involved (1 per animal)	0	2 = 28%	1 = 25%	3 = 37%	6 = 22%
Total No. of joints involved, excluding "homologous sensitized" knees	4 = 4%	17 = 22%	15 = 34%	39 = 44%	75 = 25%

In considering the total of all the experiments the knees injected with homologous strains showed only a slightly higher percentage of involvement than did other joints, and this can be accounted for largely by the figures in Experiment 3. If a definite hypersensitiveness of the joints had been induced by the experimental conditions a much higher proportion of "specifically sensitized" joints should have been inflamed.

It is impossible to coordinate our results with the conclusions of the workers previously quoted. As already mentioned, our analysis of Faber's experiments leads us to different conclusions from those that he drew; we feel, therefore, that his experiments and ours are more or less in accord. Herry states that he inoculated rabbits intravenously 8 to 15 days following intraarticular injection. Our measurements and observations of the rabbits' knees indicate that the inflammation incited by the intraarticular injection often had not completely subsided by the 12th to 15th day. It is not difficult to understand how rabbits with acutely inflamed knees, when inoculated intravenously, might have an increase of the inflammation in these joints. We cannot agree, however, that these streptococcus extracts are markedly toxic, or that intraarticular injection of them so alters the tissues of the joints that streptococci injected intravenously localize only in the treated joints.

Our experiments have nothing to do with the relation of an arthritis to a general allergic state. It is conceivable that an animal which is hypersensitive to a foreign protein or allergic to a certain bacterium, such as the tubercle bacillus, might have a severe arthritis if the foreign protein, specific bacillus, or bacterial protein were introduced into the joint. The arthritis in this event would be of the same nature as a local inflammation caused by the introduction of the specific antigen into the conjunctival sac or into the subcutaneous tissue. A discussion of a possible relation between this type of reaction and the manifestations of rheumatic fever is beyond the bounds of this article.

CONCLUSIONS.

It was impossible to demonstrate a condition of specific joint sensitization to non-hemolytic streptococci by first injecting the joints of rabbits with small doses of killed non-hemolytic streptococci, or with extracts of these organisms, and subsequently inoculating the rabbits intravenously with homologous living bacteria. Joints so treated were no more liable to involvement than were other untreated joints of the same animals.

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A NEW GENUS OF NEMATODES FROM THE DOMESTIC RABBIT.

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PLATE XI

In rabbits maintained for experimental purposes at this Department, there has been collected from the stomach on a number of occasions a nematode showing certain affinities with the genus *Graphidium* Railliet and Henry, 1909. The parasite in question, however, differs from the type species of that genus in characters which must be regarded of more than specific value and it has therefore been assigned to a new genus. It has only recently been noticed in our rabbits. I am indebted to Dr. Smith for calling my attention to it. Whether the parasite had heretofore been overlooked in autopsy examinations or has been recently introduced cannot be said. It seems probable that it originated from a wild host and is indigenous to this continent. It was first noticed after our rabbits had been transferred to quarters where they had access to the ground.

The parasite falls in the family Trichostrongylidae Railliet, 1915, and the subfamily Trichostrongylinae Leiper, 1908. The name proposed for it is *Obeliscus cuniculi.*

Generic Diagnosis.–*Obeliscus:* Head nude. Cuticle marked by a limited number of prominent longitudinal striae. Cervical and prebursal papillae present. Bursa deeply incised to form two lateral lobes. A small, distinct, well defined dorsal lobe, supported by a dorsal ray. Rays of lateral lobe fall into the usual ventral, lateral and dorsal groups. The ventro-ventral and latero-ventral rays after a pronounced divergence terminate near each other. The medio- and postero-lateral rays originate in the same stalk and are parallel. The spicules are cleft distally, the branches terminated by a recurved hook. Vulva toward posterior end of body. Well developed muscular ovijectors are present.

DESCRIPTION OF THE SPECIES.

The worms are whitish in colour but show some dark streaking due to the colour of the intestine. The anterior end is blunt. The cuticle is very finely striated transversely and marked by prominent straight or undulating longitudinal striae numbering approximately 27 to 36. Two short, stout, backwardly directed cervical papillae are present in the form of bluntly pointed spines. There is no pharynx. The oesophagus after a very slight initial reduction in diameter gradually expands to the broad posterior end where it is rounded off (Pl. XI, fig. 1).

The Male.—The length ranges from 10 to 14 mm. The maximum width occurs toward or at the posterior end. It was 211 and 229 μ in two instances. The body tapers gradually to the anterior end. The head measured 57 μ broad. The cervical papillae were located 581 μ from the anterior end. The number of longitudinal striae of the cuticula as determined near the middle of the body is approximately 27. There are a pair of prebursal papillae. The measurement of the oesophagus in two instances was 810 and 827 μ long and 103 and 90 μ in maximum breadth respectively. The spicules are brown in colour, nearly straight (Fig. 2), equal in length, and in two males measured 440 and 475μ long and 27 μ broad. Very close to the proximal end they are provided with a thin, rounded concave expansion projecting at an angle to the axis of the spicule, and at the distal end they are cleft for a short distance. One branch is flattened and enfolds the other laterally and ventrally ending in an inwardly directed recurved hook from the summit of which projects a relatively broad process rounded at the end. The other branch ends in a recurved hook directed dorsally, from the crest of which projects a very fine curved spine (Fig. 3). The bursa (Fig. 4) consists of two large, rounded, lateral lobes and a small dorsal lobe. The internal surface of the former, except at the periphery, is thickly covered with small round papillae variable in size. Peripherally the same surface is marked by radiating ridges which may show branching. Each lateral lobe is supported by six well-developed rays. The ventro-ventral ray is small and bends slightly ventral. It originates from the base of the latero-ventral ray. The latter is large and after passing backward for more than half its length curves ventral and terminates near the end of the former. The externo-, medio-, and postero-lateral rays form the usual lateral system. The former is large and runs along the middle of the lobe terminating near its margin. The other two are small, lie close together, and bend slightly dorsal. The externo-dorsal ray is small and slightly curved.

The dorsal lobe of the bursa (Fig. 5) is median, distinctly demarked from and lies within the lateral lobes. It may be looked on as slightly three-lobed, there being two lateral and a very small terminal median lobe. The end of the dorsal ray extends into the latter where it divides into two branches, each of which bifurcates, the external portions being the shorter. A little anterior to the first division two lateral spurs are given off (Fig. 4).

The Female.—It ranges in length from 15 to 18.5 mm. The maximum width (387 and 546 μ in two instances) occurs at the middle of the body. The width of the head is 119 μ. The tail is pointed. From the middle region the body gradually tapers in both directions but a few millimetres (3.2 to 3.6) from the posterior end there is an abrupt reduction in diameter (Fig. 6). In a specimen measured this region had an anterior and middle diameter of 264 and 282 μ respectively. The vulva is a transverse slit located at the anterior end of this region. In two measurements made the anus was located 226 μ and 299 μ from the posterior end. The cervical papillae were located 649 μ from the anterior end. The longitudinal striae of the cuticula number approximately 32 to 36. The oesophagus measured 1 mm. long. The diameter near the anterior end was 37 μ and the posterior diameter in two specimens 131 μ and 144 μ. The nerve ring occurred 405 μ from the anterior end. The vagina is short, leading directly inward into well-developed muscular ovijectors (Fig. 6). The terminal portions of the uteri enter the ovijectors anteriorly and posteriorly. This region of the uterus is separated from the rest by a constriction, is somewhat narrower and appears to be specialised for passing the ova into the ovijector.

The ova are elliposoidal in shape (Fig. 7). They are provided with a very thin, double-contoured shell. When deposited they are in the morula stage. In size they range from 76 to 86 μ long and 44 to 45 μ broad.

This species shows certain relations to *Graphidium strigosum* (Duj. 1845) Railliet and Henry, 1919, type species of *Graphidium*, reported from Europe in rabbits (*Oryctolagus cuniculus* and *Lepus europaeus*). The more striking structural differences of the latter are as follows (1): The spicules are long, filiform, and frayed out at the distal end. There is no dorsal lobe to the bursa. The five rays of the lateral and ventral systems are uniformly and symmetrically spaced, and the externo-dorsal ray is long and prominent. These differences are considered sufficiently important to warrant placing the form here described in a new genus.

REFERENCE.

(1) Hall, M. C. (1916). Nematode parasites of mammals of the orders Rodentia, Lagomorpha and Hyracoidea. *Proc. U. S. Nat. Mus.*, l, 1–258.

EXPLANATION OF PLATE XI.

All drawings except Figs. 3 and 5 were made with a camera lucida.

Fig. 1. Anterior end of a male, showing oesophagus and cervical papillae. × 103.

Fig. 2. Spicules. × 170.

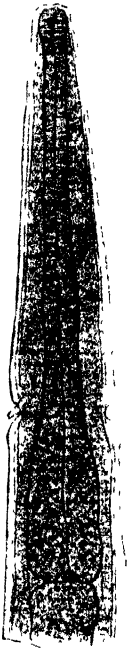
Fig. 3. End of a spicule showing the two branches.

Fig. 4. Dorsal view of bursa. Shape of dorsal lobe distorted. × 80.

Fig. 5. Outline of dorsal lobe of bursa showing dorsal ray.

Fig. 6. Body of female in region of vulva showing beginning of narrow posterior portion of the body, muscular ovijectors, uteri, ovaries, and intestine. × 103.

Fig. 7. Drawing of ovum when deposited. × 680.



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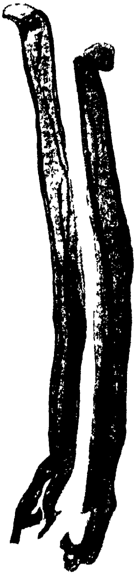

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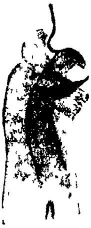

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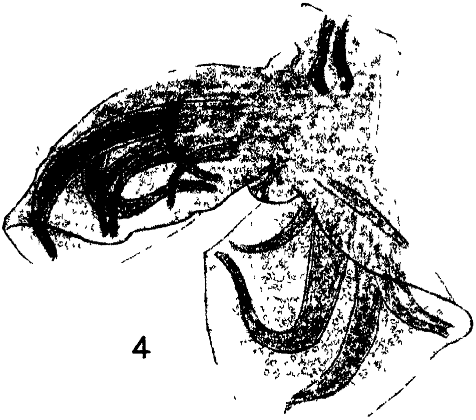

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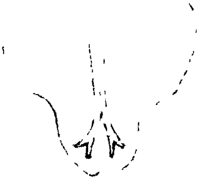

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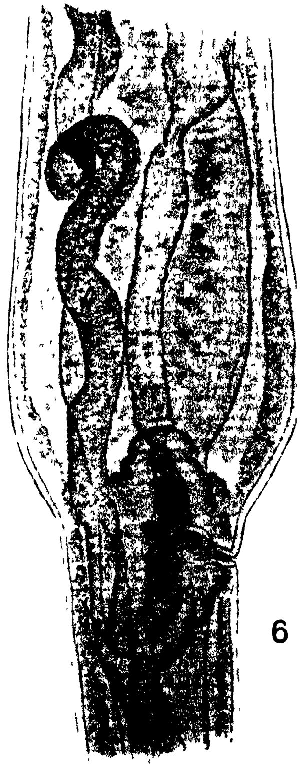

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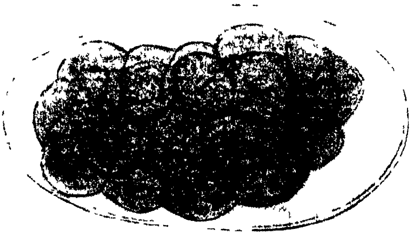

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